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Protease Inhibitors of the Eastern Oyster, *Crassostrea virginica*, and their Relationship to the Protozoan Pathogen, *Perkinsus marinus*

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PROTEASE INHIBITORS OF THE EASTERN OYSTER,
CRASSOSTREA VIRGINICA, AND THEIR RELATIONSHIP
TO THE PROTOZOAN PATHOGEN, *PERKINSUS MARINUS*

A Thesis
Presented to
The Faculty of the School of Marine Science
The College of William and Mary

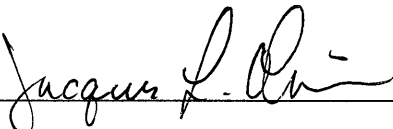
In Partial Fulfillment
Of the Requirements for the Degree of
Master of Science

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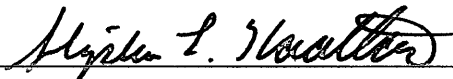
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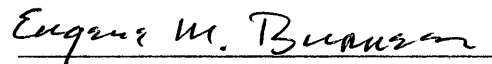


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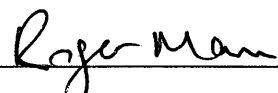
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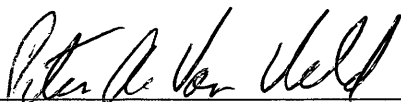
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DEDICATION

For W. M. Oliver

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General Abstract

Eastern oysters, *Crassostrea virginica*, possess numerous humoral defense factors. They include lectins, lysosomal enzymes, the prophenoloxidase-activating system, acid phosphatases, and stress proteins. Recent attention has focused on the role of protease inhibitors in humoral defense against Dermo, a disease caused by the oyster pathogen, *Perkinsus marinus*. Protease inhibitors have been described in numerous vertebrates and invertebrates. They serve as important regulatory molecules and as potent mechanisms of neutralizing pathogen virulence factors. In this study, low molecular weight (LMW) protease inhibitory activity (PI) was detected in the plasma of eastern oysters. The PI was determined to be specific for extracellular proteases of *P. marinus* and was capable of preventing the proteolytic digestion of a specific oyster plasma protein of 35 kDa. The role of LMW PI in disease resistance was then posed. This led to the analysis of LMW PI in ten selectively bred families of oysters exhibiting varying degrees of *Haplosporidium nelsoni* (MSX) and *P. marinus*-resistance. Results showed a correlation of PI with survival and with disease intensity. The higher surviving families exhibited higher PI on average and more heavily infected oysters exhibited lower PI. Additionally, an F4 generation of MSX-resistant oysters displaying resistance to Dermo was compared to two stocks of oysters endemic to the Chesapeake Bay in terms of PI, survival, and average disease intensity. There was no correlation between PI and survival; however, a weak correlation similar to the selected families was observed between PI and disease intensity. The question of PI dynamics during *P. marinus* infection progression was also posed. Susceptible oysters were experimentally challenged with live and killed *P. marinus* and PI monitored over a five week period. Results indicate that PI was inducible

in both treatments at week 1 post-challenge (p.c.), however, oysters challenged with live *P. marinus* continued to show decreases in PI at week 5 p.c. In contrast, PI stabilized in the treatment challenged with killed *P. marinus* at week 5 p.c. Results of the four experiments suggest that LMW protease inhibitors play a role in conferring resistance to higher infection intensities of Dermo.

Chapter 1

Analysis of the Effects of *Perkinsus marinus* Proteases on Plasma Proteins of the Eastern (*Crassostrea virginica*) and Pacific Oyster (*Crassostrea gigas*)

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Abbreviations: PBS phosphate buffered saline, SDS-PAGE sodium dodecyl sulfate-polyacrylamide gel electrophoresis, kDa kilodalton, ppt part per thousand, T-TBS Tween-tris buffered saline, PVDF polyvinylidene difluoride, ECL enhanced chemiluminescence, pI isoelectric point, IEF isoelectric focusing, BCA bicinchoninic acid, DTT dithiothreitol

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Abstract

We employed two *in vitro* buffer systems to determine the potential pathogenic effects of *Perkinsus marinus* serine proteases on the plasma proteins of the eastern oyster (*Crassostrea virginica*) and the Pacific oyster (*Crassostrea gigas*). Specifically, this study characterized the oyster plasma protein targets of *P. marinus* proteases. Additionally, protease-specific inhibitory activity was revealed upon comparison of artificial (PBS) and endogenous (plasma-based) diluents employed during protease digestions. It was found that a *C. virginica* plasma protein of approximately 35 kDa was eliminated when a standard buffer (PBS) was used as a diluent; however, this protein was preserved when a low-molecular weight, plasma-based, diluent was used. The results strongly indicate that low-molecular weight inhibitors of *P. marinus* proteases are present in oyster plasma. A control (non-parasitic) serine protease, α -chymotrypsin, was employed to ascertain the specificity of the protease inhibitors. Although α -chymotrypsin possesses ample proteolytic activity for *C. virginica* plasma proteins, the anti-proteases could only specifically inhibit *P. marinus* proteases. Such specificity of anti-protease activity is not uncommon among low-molecular weight serine proteases. The hemolymph target protein was isolated by 2D electrophoresis and the isoelectrically isolated for further characterization by N-terminal amino acid sequencing.

Introduction

Perkinsus marinus is a protozoan pathogen (Apicomplexa, Levine, 1978) that is the causative agent of Dermo, a lethal disease afflicting the eastern oyster (*Crassostrea virginica*) (Andrews, 1988). Although aspects of the disease process such as adverse physiological effects on the eastern oyster (Paynter, 1996) and abiotic effects on the susceptibility, transmission, and infectivity of *P. marinus* (Chu, 1996) have been addressed, little is known of the pathogenic mechanisms employed (Anderson, 1996). Such knowledge is necessary for designing therapeutic and prophylactic modalities.

Successful *in vitro* propagation of the pathogen, as described by La Peyre et al. (1993), has provided the means to isolate and analyze the virulence factors of *P. marinus*. Among the *in vitro* secreted extracellular proteins of *P. marinus*, La Peyre and Faisal (1995) discovered chymotrypsin-like serine proteases. They demonstrated that these proteases were capable of digesting a variety of proteins including hemolymph proteins. As with many other pathogens (McKerrow, 1989, McKerrow et al., 1993) the possibility that these proteases could also facilitate invasion, compromise host defense mechanisms, and provide the invading pathogen with suitable nutrients has been explored. In this context, it was demonstrated that *P. marinus* proteases can compromise immune defense mechanisms of the eastern oyster (Garreis et al., 1996) and favor the protozoan's propagation in oysters (La Peyre et al., 1996). Identification and characterization of putative molecular targets of *P. marinus* proteases, however, has yet to be performed.

One impediment to these studies has been the general dearth of knowledge regarding the protein composition of oyster plasma.

Of additional importance to *P. marinus* pathogenesis research is the fact that Pacific oysters (*Crassostrea gigas*) contract only light infections upon exposure. Meyers et al. (1991) found that prevalences and mortalities in diploid and triploid *C. gigas* exposed to *P. marinus* were lower than that in diploid and triploid *C. virginica*. This suggested a tolerance of *C. gigas* to *P. marinus*, however, the exact mode of resistance of Pacific oysters to Dermo remains to be elucidated. Meyers et al. (1991), however, postulated that the pathogen might be unable to proliferate in *C. gigas*. In fact, La Peyre (1993) noted the lower hemolymph protein concentrations in Pacific oysters may pose a nutritional limitation for the pathogen. Alternatively, *C. gigas* might possess specific defense mechanisms that allow the host to eliminate the pathogen.

This study was conducted to determine which plasma components may be the primary targets of *P. marinus* proteases in *C. virginica* and *C. gigas*. Such information may be critical in revealing the possible molecular basis of *Perkinsus* pathogenesis. Paramount to such studies is the development of a protease assay system that simulates *in vivo* physio-chemical conditions. It was found that when an artificial buffer, PBS, was employed in the protease assay system a distinctly different digestion pattern of plasma proteins is observed than that observed with an endogenous, plasma-based diluent. Based on these observations, the existence of low-molecular weight protease inhibitors in oyster plasma was revealed and that these inhibitors were protease specific.

Materials and Methods

Oysters

Eastern oysters (shell height approximately 70-80 mm) were purchased from Permaquid Inc., Waldoboro, Maine and acclimated for three months in filtered York River (Virginia) water. Oysters were notched and approximately 3-4 ml of hemolymph were bled from the adductor sinus using a syringe and 25 gauge needle. Hemolymph was then filtered using a 0.45 μ m filter (Costar Scientific Corp., Cambridge, MA) and aliquots stored in sterile microfuge tubes at -80°C until further use. Oyster plasma was assayed for protein determination using BCA protein assay kit (Pierce, Rockford, IL).

Pacific oysters, *Crassostrea gigas*, were obtained from Lookout Point Oyster Company (Lookout Point, Oregon). Oysters were notched and bled immediately upon arrival as described above. Hemolymph was sterile filtered using 0.45 μ m filter (Costar) and aliquots stored at -80°C until further use. Protein determinations of plasma were determined using the BCA assay (Pierce).

Perkinsus marinus Extracellular Protein (ECP) Preparation

Perkinsus marinus -1 cells were cultured for 8 weeks in JL-ODRP-1 medium (La Peyre et al., 1993). Culture supernatants were obtained by centrifuging the cultures at 3600 x g in 50 ml centrifuge tubes at 4°C for 20 minutes. The supernatants were decanted into Centriprep-10 concentrators (Amicon Inc., Beverly, MA) and centrifuged

at 3600 x g for 40 minutes at 4°C. After centrifugation, cold (4°C) PBS (137 mM NaCl, 3 mM KCl, 8 mM Na₂HPO₄, 2 mM KH₂PO₄, pH 7.2) was added to the Centriprep-10 apparatus and the centrifugation step repeated. This procedure was performed three times to equilibrate the ECP in PBS. ECP was then filtered using a 0.22 µm bottle filter system (Costar) and stored at 4°C until further use. Protein determination of concentrated ECP was performed using the BCA protein assay (Pierce).

Detection of Protease Activity

A qualitative analysis of concentrated ECP was performed to detect and confirm the proteolytic activity of *P. marinus* proteases. The proteolytic activity of ECP was analyzed using substrate (gelatin) gel electrophoresis as described by La Peyre et al. (1995).

Production of *C. virginica* Plasma Protein Antisera

Female New Zealand rabbits (2.27 kg) (Franklin Rabbitry, Wake Forest, NC) were immunized with 0.45 ml of 1:1 emulsion of 200 µg *C. virginica* plasma/ml PBS and Freund's complete adjuvant (Sigma Chemical Company, St. Louis, MO). Fifty microliters of the emulsion were injected intramuscularly into the hind legs and 4, 100 µl subcutaneous injections along the back were administered. Rabbit serum was procured from ear vein bleeds at 2 and 4 months post-injection. Aliquots were frozen at -80°C until used. Antisera were tested for reactivity with eastern and Pacific oyster plasma through immunoblot analysis. Results showed cross-reactivity of rabbit antisera to all

observable proteins in both species of oysters and no cross-reactivity with *P. marinus* protein.

Incubation of Oyster Plasma With *P. marinus* Proteases and α -Chymotrypsin

Ten micrograms of concentrated ECP were placed in a sterile microfuge tube at a volume of 14 μ l. Ten micrograms of *C. virginica* oyster plasma proteins were added to the same tube in a volume of 2 μ l. The reaction mixture was brought to a final volume of 100 μ l using either PBS or a plasma-based diluent. The latter diluent was generated by centrifuging oyster plasma in a Centricon-10 concentrator (Amicon Inc.). The filtrate (containing <10 kDa proteins) was retained and used as the plasma-based diluent. The pH of the PBS reaction mixture and the plasma-based diluent were 7.2 and 7.4, respectively. The osmolarity of the reaction mixtures was measured using a Westcor 5500 Vapor Pressure Osmometer (Westcor, Inc., Logan, Utah). The osmometer was calibrated using 100 and 1,000 mmol/kg standards. Ten microliters of each mixture was loaded into the osmometer and measured. The osmolarity of the PBS reaction mixture and the plasma-based diluent was 316 mmol/kg and 476 mmol/kg, respectively. The reaction mixture was capped and covered with aluminum foil and placed in an end-over-end rotator for 24 hours at 17°C. In separate tubes negative controls consisting of an aliquot of ECP and an aliquot of oyster plasma were similarly placed on the rotator and incubated for the same duration.

Ten microliters of *C. gigas* plasma protein corresponding to 10 μ g of protein were added to the microfuge tube. Ten micrograms of concentrated ECP were placed in a sterile microfuge tube at a volume of 13.7 μ l. The reaction mixture was brought to a final

volume of 100 μ l using PBS or the *C. gigas*-derived plasma-based diluent generated as previously described. Negative controls were unmixed ECP and *C. gigas* plasma incubated in separate microfuge tubes. The pH of the PBS and plasma-based diluent reaction mixtures were 7.2 and 7.4, respectively. Osmolarity of the two reaction mixtures was 407 mmol/kg for the PBS diluent and 854 mmol/kg for the plasma-based diluent. Osmolarity measurements were made as previously described. Incubations of the mixture and controls were performed as previously described.

α -Chymotrypsin from bovine stomach (Sigma) was incubated with eastern oyster plasma proteins using the methods previously described. Briefly, 10 μ g of α -chymotrypsin diluted in PBS were incubated with 10 μ g of eastern oyster plasma proteins. The remaining volume of the reaction mixture was filled with either PBS or the plasma-based diluent. Analysis of targets was performed using SDS-PAGE, immunoblotting, and colloidal gold staining procedures described above

Identification of Protease Targets in Oysters

After the protease incubation, an aliquot from each reaction mixture was electrophoresed in a reducing 12% SDS-polyacrylamide gel (Harlow and Lane, 1988) using a mini-PROTEAN II Cell apparatus (Bio-Rad Laboratories, Hercules, CA). All samples were prepared in reducing (DTT) sample buffer as described by Harlow and Lane (1988). The volume of the reaction mixture loaded onto the gel was equivalent to that which would contain 1 μ g of oyster plasma protein. Controls (plasma incubated without ECP and ECP alone) were analyzed in the same manner. Biotinylated broad range molecular weight markers (Bio-Rad) were loaded as a reference. After

electrophoresis proteins were transblotted at 100 V for 1 hour to PVDF membranes (Millipore Corp., Burlington, MA) as described by the Mini Trans-blot Electrophoretic Cell Manual (Bio-Rad) and immunoblotting analysis was performed to more readily identify oyster plasma protein targets. Following transblotting, membranes were blocked with T-TBS (0.1% (v/v) Tween20, 25mM Tris, 137 mM NaCl, 3 mM KCl, 8 mM Na₂HPO₄, 2 mM KH₂PO₄, pH 7.2) (Arkoosh and Kaattari, 1990) overnight at 4°C. Membranes were incubated on a shaker at room temperature with a rabbit antiserum generated against *C. virginica* plasma proteins (diluted 1:5,000 in T-TBS) for 1.5 hours. Membranes were washed 3 times in T-TBS at 5 minutes per wash. Membranes were incubated with a goat-anti-rabbit-HRPO antibody (Sigma Chemical Company) (diluted 1:5,000 in T-TBS) and streptavidin-horse radish peroxidase (diluted 1:1,000 in T-TBS) for molecular weight marker identification for 1 hour at room temperature. Membranes were washed five times in T-TBS. ECL reagents (Amersham Life Science, Buckinghamshire, England) were used for chemiluminescent development of the membranes on ECL Hyperfilm (Amersham Life Science). Following ECL development, membranes were rinsed 10 times with deionized-distilled water and total protein stained with Colloidal Gold Stain (Bio-Rad). Target protein molecular weight determination was performed via comparison to molecular weight standards using densitometric analysis (Integrated Separation Systems Enprotech, Natick, MA).

Two-Dimensional Isoelectric Focusing and Western Blot of Eastern Oyster Plasma Proteins

A two-dimensional IEF analysis was performed to determine potential pI diversity of the 35 kDa target in the eastern oyster plasma previously identified in the above analyses. First dimension IEF tube gels were prepared and samples electrophoresed according to the methods described by Harlow and Lane (1988) using the Bio-Rad Minigel apparatus. Each tube gel was layered upon 12% SDS-polyacrylamide gels for second dimension electrophoresis. In order to attain reducing conditions for the tube gel, 200 μ l of reducing (DTT) sample buffer prepared according to Harlow and Lane (1988) was added on top of the tube gel. After second dimension electrophoresis was performed, proteins were transblotted to PVDF membranes, immunoblotted, and stained by the methods previously described.

N-Terminal Amino Acid Sequencing and Amino Acid Composition of Eastern Oyster Target Proteins

Five micrograms of target protein were isolated via 2D IEF as previously described. Preparation of the membrane for N-terminal sequencing and amino acid analysis was performed using a protocol (Gilardi, M. L., 1996) provided by Commonwealth Biotechnologies, Inc. (Richmond, VA). Briefly, following transblot of proteins to a PVDF membrane, the membrane was stained with 0.1% (v/v) Coomassie Brilliant Blue R-250 in 50% (v/v) methanol/deionized-distilled water for 5 minutes at room temperature. Membranes were destained with 50% methanol, 10% acetic acid, 40% deionized-distilled water for 10 minutes at room temperature. Membranes were rinsed

twice with deionized-distilled water, equilibrated for 10 minutes in deionized-distilled water, and then air dried for 1 hour. The target protein band was excised from the membrane and sent to Commonwealth Biotechnologies, Inc. for N-terminal amino acid sequencing and amino acid composition analysis. Sequence information was compared to known sequences by searching SWISSPROT using BLASTP (2.0.8). The Expect value (E) in the database search was increased to 1,000 to provide statistically significant or non-significant matches.

Results

Oyster Plasma Protein Digests Using PBS and Plasma-Based Diluents PBS diluent

Co-incubation of *C. virginica* plasma proteins with *P. marinus* proteases using a PBS diluent resulted in the complete elimination of a 35 kDa protein band (Figures 1a & 1b, lane 3 – arrow). An immunoblot using a rabbit anti-*C. virginica* plasma permitted a clearer resolution of the digested oyster “target” protein (Figure 1a, lane 4 – arrow). It should be noted that an additional band appears upon digestion (Figure 1a, lane 3 – asterisk). It is unlikely that this band represents cross-reactivity with *P. marinus* ECP since no such band is apparent in lane 2. The appearance of this band suggests that it may be a product of partial proteolytic digestion of an, as yet to be determined, high molecular weight species by ECP.

Plasma-based diluent

Use of a physiologically relevant plasma-based diluent revealed a strikingly different digestion profile from that observed with PBS. Under these conditions the 35 kDa protein band remains substantively intact (Figures 1c & 1d, lane 3 – arrow). Although the 35 kDa protein appears protected in this diluent, some partial digestion appears to be occurring, as new, low molecular weight species are generated (Figures 1c & 1d, lane 3 – asterisks).

Plasma proteins from the relatively *P. marinus*-resistant oyster, *C. gigas*, were exposed to *P. marinus* proteases under identical conditions used with *C. virginica* (Figure 2). In this case, the PBS diluent did not support the complete digestion of any plasma proteins, however, partial digestion appears to be occurring with a protein of approximately 66 kDa (Figures 2a & 2b, lane 3 – circle). In addition, some breakdown products of approximately 35 kDa are observed (Figures 2a & 2b, lane 3 – brackets) which may be due to degradation of high molecular weight proteins. In the presence of the plasma diluent a more intense digestion of the higher molecular weight species (approximately 66 kDa) (Figures 2c & 2d, lane 3 – circle) occurs, leaving an assortment of lower molecular weight species (Figures 2c & 2d, lane 3 – bracket). However, digestion is not as thorough as with *C. virginica* proteins.

Effects of α -Chymotrypsin on Eastern Oyster Plasma Protein Substrates

Incubation of eastern oyster plasma with a non-*Perkinsus* (bovine) serine protease, α -chymotrypsin, in PBS resulted in proteolysis of the 35 kDa target as was demonstrated with *P. marinus* serine proteases (Figures 3a & 3b, lane 3 – arrow). In addition to the

elimination of the 35 kDa target, a 30 kDa band becomes apparent (Figures 3a & 3b, lane 3 – asterisk). It is likely that the 30 kDa band represents degradation products of the 35 kDa target protein.

When the diluent was switched from PBS to the plasma-based diluent, the result was unchanged. Eastern oyster plasma proteins were digested (Figures 3c & 3d, lane 3 – arrow). In addition, there appear to be degradation products at 30 kDa (Figure 3c, lane 3 – asterisk) as seen in Figures 3a and 3b, lane 3.

Two-Dimensional Isoelectric Focusing of Eastern Oyster Plasma Proteins

Further characterization of the plasma proteins by two-dimensional isoelectric focusing (2D IEF) and immunoblotting revealed a rather restricted pI heterogeneity for the 35 kDa target protein (Figure 4a). Specifically, the 35 kDa target of the eastern oyster shows no pI heterogeneity and the target is composed of a single protein (Figures 4a & 4b – circle/arrow). Identification of the target can be determined upon comparison of the 2D IEF of oyster plasma exposed to ECP (Figures 4c & 4d – circle/arrow) and the oyster plasma control (Figure 4c, lane 2 – arrow). Comparison of figures 4a and 4c reveals elimination of a second target near 66 kDa not previously seen in one-dimensional immunoblotting (Figures 4a & 4c – circle/asterisk). The isoelectric point range for the 35 kDa target is 5.9 – 6.6.

A second 2D IEF of oyster plasma proteins was performed. Proteins were transblotted and non-covalently stained. N-terminal amino acid sequencing and amino acid composition analysis revealed the first 10 amino acid residues (Table 1). Amino acid composition analysis revealed the target to be abundant in serine, glycine, and

alanine (Table 2). Database searches were performed to match the obtained sequence with other known amino acid sequences. Results revealed best match to be with plant plasma membrane ATPases (50% identity), however these matches were considered to be statistically non-significant due to the high Expect values (E) of the returned matches (data not shown).

Discussion

This study was conducted to analyze the *in vitro* pathogenic effects of *P. marinus* proteases on plasma proteins of two oysters, *C. virginica* and *C. gigas*. When *C. virginica* proteins were incubated with *P. marinus* ECP, degradation of a 35 kDa protein was observed. One-dimensional SDS-PAGE of host plasma proteins allowed us to characterize the molecular weight of the target, however, two-dimensional isoelectric focusing and electrophoresis was required to determine the possible isoelectric heterogeneity of proteins. The isoelectric diversity of the target appears to be limited and consisting of one or possibly two proteins.

Our experiments were designed to determine any plasma protein targets of *P. marinus* proteases, however, we were able to additionally identify a potentially novel host defense factor in *C. virginica* plasma. Initial *in vitro* experiments with host plasma proteins and *P. marinus* ECP in PBS (pH 7.2) showed consistent digestion of host proteins. Later experiments employed a plasma-based diluent derived by removing large (>10 kDa) molecular constituents of the plasma. This fraction was used in place of PBS as the reaction mixture diluent to determine if an endogenous diluent would reveal comparable effects as PBS. Under these conditions preservation of the host target protein

occurred. The most likely reason for this lack of activity in the plasma-based diluent would be the supplementation of protease inhibitors. Analysis of the <10 kDa plasma diluent with 8 kDa pore size membrane resulted in retention of the inhibitory activity (data not shown). This indicates that the inhibitory activity resides with 8-10 kDa sized molecule(s) and cannot be due to factors such as minor differences in pH, osmolarity, or other aspects of ion or low molecular weight composition. Furthermore, evidence that osmolarity of each diluent (PBS, *C. virginica*-derived or *C. gigas*-derived) was not a factor affecting proteolytic activity is the observation that expression and activity of *P. marinus* proteases occurs in a culture medium with elevated osmolarity (650 mmol/kg) (La Peyre et al., 1993; La Peyre et al., 1995). Further evidence that pH was not a factor affecting proteolytic activity is the finding that *P. marinus* protease activity occurs in a variety of buffers with varying pH (5.0-9.5), including PBS (La Peyre et al., 1995). Thus, if plasma protease inhibitors are operative, it may be that dilution of plasma in PBS may effectively reduce endogenous inhibitors to a concentration insufficient to block the activity of *P. marinus* proteases. Protease inhibitors are known to be important regulatory molecules as well as having importance in humoral defense (Ellis, A., 1987; Calkins and Sloane, 1995; Wiedow et al., 1998). Furthermore, protease inhibitors have been identified in a number of marine invertebrates (Donovan and Laue, 1991; Sousa, M. et al., 1992) and specifically in molluscs (Bender et al., 1992; Thorgersen et al., 1992; Armstrong and Quigley, 1992).

La Peyre et al. (1995) noted the similarity of *P. marinus* proteases to chymotrypsin-like enzymes. Incubations of eastern oyster plasma with another serine protease, α -chymotrypsin, were undertaken to compare specificity *P. marinus* proteases and α -

chymotrypsin for plasma proteins. Additionally, the specificity of the inhibitory activity in the <10 kDa fraction of eastern oyster plasma against α -chymotrypsin was examined. Results showed that α -chymotrypsin can recognize and proteolytically digest eastern oyster plasma proteins. Thus, *P. marinus* serine proteases and α -chymotrypsin share substrate specificity. However, whereas *P. marinus* proteases were successfully inhibited from digesting oyster proteins with the endogenous inhibitor, α -chymotrypsin was uninhibited. This differential sensitivity among serine proteases is common. For example, La Peyre et al. (1995) showed that *P. marinus* proteases exhibited low activity in the presence of PMSF and chymostatin, but were quite active in the presence of other serine protease inhibitors such as 3,4-DCI and elastatinal. Additionally, Faisal et al. (1998) suggested that the ability of *C. gigas* to strongly inhibit *P. marinus* proteases yet weakly inhibit trypsin was due to specificity of protease inhibitors in *Crassostrea* spp. Thus, it appears that inhibitory activity of the eastern oyster is specific and may have developed and been directed against *P. marinus* proteases.

Pacific oysters have been of particular interest due to their ability to resist the *P. marinus* upon challenge (Meyers, 1991). Having observed the patterns of susceptibility and resistance of eastern oyster plasma proteins to *P. marinus* proteases, it was of interest to determine the effects on Pacific oyster plasma proteins. Results demonstrated that *C. gigas* plasma proteins are susceptible to proteolysis in *both* diluents. Despite the apparent partial digestion of an approximately 66 kDa protein in Pacific oyster plasma, the sensitivity of this protein appears to be less than the sensitivity of the p35 target in the eastern oyster, as p35 was completely eliminated under identical conditions. Although there appears to be a target of *P. marinus* proteases within Pacific oyster plasma, there

are a number of possibilities that may preclude disease susceptibility being contingent upon the loss of this target. The 66 kDa target may be inconsequential to any vital process in the Pacific oyster. Alternatively, the incomplete proteolysis of the protein suggests that the protein is only slightly sensitive to *P. marinus* proteases and full elimination of this protein does not occur. Finally, there may be mechanisms utilized by Pacific oysters outside the scope of this *in vitro* experiment that prevent *P. marinus* from exerting its virulence via proteases. This may occur by suppression of protease expression or perhaps rapid clearance of the pathogen before replication can occur.

The possible function of the target molecule (p35) within *C. virginica* hemolymph may be most readily ascertained by identification of structurally homologous molecules. Therefore, as the p35 protein was isoelectrically restricted, its isolation and analysis was readily accomplished. Amino acid composition analysis revealed p35 to be abundant in serine, glycine, and alanine. Furthermore, the N-terminal amino acid sequence of the first 10 residues of p35 was obtained. The closest sequence identity (50%) was found with plant plasma membrane ATPases when a search was conducted to ascertain homologous sequences, thus the information gained from the database searches has provided limited insight into the possible function of the p35 target. Although sequence matches were identified, it is likely that such matches were merely random occurrences. This is evident by the high E values (428, 561, and 735) of each match, which signified low statistical significance.

Additional information, albeit limited, can be gleaned from the amino acid composition of p35. The abundance of alanine, an uncharged amino acid with a non-aromatic side chain, in p35 is evidence that supports p35 as a specific target of *P.*

marinus serine proteases. Kraut (1977) described elastase, another common serine protease, as having preference for alanine residues. Elastase was 4000 times more efficient in hydrolyzing peptide substrates with amino acid sequences that contained consecutive alanine residues. Further inferences about the function and identity of p35 through amino acid composition cannot be made.

Despite the limited effectiveness of the amino acid analyses for protein identification, the unique combination of molecular weight and pI are suggestive that p35 might be a subunit of a serum lectin. Vasta et al. (1984) affinity purified a serum lectin of *C. virginica* using rat red blood cells (rRBC) and analyzed it via reducing SDS-PAGE. Their results showed three subunit bands, OSLS-I (67 kDa), OSLS-II (34 kDa), and OSLS-III (25 kDa). It is interesting to note the presence of a 34 kDa (OSLS-II) protein identified by Vasta et al. And our findings of a 35 kDa target in oyster hemolymph. Additionally, Vasta et al. (1984) isoelectrically focused the serum lectin and found that the lectin had a broad pI range (pH 5-6 and pH 7-8) with slightly greater heterogeneity than our 35 kDa target. The affinity purified lectin was also tested for its ability to agglutinate protease-treated rat, horse, and human O RBC. Immediate comparisons can be made between the two molecules. They both have a similar molecular weight and have close pI ranges. However, caution should be taken before any conclusions can be drawn between the two molecules. It is possible that OSLS-II and p35 merely share physical characteristics, but functionally they are distinct. Additionally, Vasta et al. (1984) did not verify other proteins in the hemolymph which were not affinity purified and may also share a similar molecular weight. To confirm OSLS-II and p35 as the same molecule, an N-terminal sequence of OSLS-II would have to be determined and

compared with that of p35. Further evidence to support homology would be to expose the serum lectin to *P. marinus* ECP. Digestion of OSLS-II would support N-terminus results that OSLS-II and p35 are the same protein and would establish that the serum lectin is a target of *P. marinus* proteases.

Confirmation of OSLS-II/p35 homology would have important implications in terms of the molecular pathogenesis of *P. marinus*. It is widely known that lectins play an important role in invertebrate humoral and cellular defense (Arason, G. J., 1996). Elimination of an oyster defense molecule used in non-self recognition by pathogen proteases would represent a pathogenic mechanism not previously described in the eastern oyster.

There is a general dearth of knowledge concerning protease inhibitors in the hemolymph of oysters. Previous studies on the serological and hemolymph composition of eastern oysters have focused on lysozyme activity, agglutinins, and hemocyte characterization and responses (Cheng and Rodrick, 1975; Chu and La Peyre, 1989; Li and Flemming, 1967; Chintala et al., 1994; Anderson et al., 1995; Anderson, 1996). However, in a recent study Faisal et al. (1998) provided evidence that *C. virginica* and *C. gigas* were capable of producing protease inhibitors. These findings support our observations that protease inhibitors are present in *C. virginica* although we could not infer the presence of protease inhibitors in *C. gigas*. Our study does not preclude the possibility that *C. gigas* can produce protease inhibitors, particularly high molecular weight inhibitors. In fact, Faisal et al. (1998) observed much higher protease inhibitory activity in *C. gigas* towards *P. marinus* proteases than *C. virginica* and speculated that this inhibitory activity could be attributed to high molecular weight species such as α_2 -

macroglobulin-like molecules. In addition, previous studies by Adham and Faisal (1997) suggested the presence of α_2 -macroglobulin-like activity in *C. virginica* and *C. gigas* plasmas. This difference in protease inhibitory activity between the two species may be important in the higher susceptibility of *C. virginica* over *C. gigas*.

Our findings implicate *P. marinus* proteases and p35 in the molecular pathogenesis of the disease. It is possible that the p35 target is vitally important to the survival of the oyster and its elimination may be important during infection. Although p35 is not digested by *P. marinus* proteases when in the presence of plasma-based diluent, it is possible that p35 can become susceptible during the progression of the disease. This might occur if the pathogen is capable of compromising host anti-proteolytic activity observed herein. The work presented here, however, is reflective of *in vitro* conditions only. *In vitro* experiments have solely allowed us to identify and partially characterize the target. Our data suggest a dynamic interplay between proteases, target proteins, and inhibitors. A fuller understanding of these potential pathogenic proteases requires *in vivo* correlation of target protein loss with *P. marinus* infections.

The experiments performed here are important first steps in understanding the role of *P. marinus* proteases in the pathogenesis of Dermo. It seems unlikely that *P. marinus* proteases are the exclusive virulence factor in Dermo, however, they may very well play a critical role in disease initiation or progression. The existence of protease inhibitors within eastern oysters suggests a previously undescribed defense mechanism.

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References

- Adham, K. and Faisal, M. 1997. Involvement of protease inhibitors in defense mechanisms of oyster: A model to study acquired immunodeficiency syndromes. *Dev. Comp. Immunol.* **21**:101.
- Anderson, R. S. 1996. Interactions of *Perkinsus marinus* with humoral factors and hemocytes of *Crassostrea virginica*. *J. Shellfish Res.* **15**:127-134.
- Anderson, R. S., Bureson, E. M., and Paynter, K. T. 1995. Defense responses of hemocytes withdrawn from *Crassostrea virginica* infected with *Perkinsus marinus*. *J. Invertebr. Path.* **66**:82-89.
- Andrews, J. D. 1988. Epizootiology of the disease caused by the oyster pathogen *Perkinsus marinus* and its effect on the oyster industry. *Am. Fish. Soc. Spec. Publ.* **18**:47-63.
- Arkoosh, M. R. and Kaattari, S. L. 1990. Quantitation of fish antibody to a specific antigen by an enzyme-linked immunosorbent assay (ELISA). In "Techniques in Fish Immunology" (J. S. Stolen, T. C. Fletcher, D. P. Anderson, B. S. Roberson, W. B. Van Muiswinkel, Eds.), pp. 15-24. SOS Publications, Fair Haven, New Jersey.
- Arason, G. J. 1996. Lectins as defence molecules in vertebrates and invertebrates. *Fish Shellfish Immunol.* **6**:277-289.
- Armstrong, P. B. and Quigley, J. P. 1992. Humoral Immunity: α_2 -macroglobulin activity in the plasma of mollusks. *Veliger.* **35**:161-164.
- Bender, R. C., Fryer, S. E., and Bayne, C. J. 1992. Proteinase inhibitory activity in the plasma of a mollusc: Evidence for the presence of α -macroglobulin in *Biomphalaria glabrata*. *Comp. Biochem. Physiol.* **102B**(4):821-824.

- Calkins, C. and Sloane, B. 1995. Mammalian cysteine prtease inhibitors: Biochemical properties and possible role in tumour progression. *Biol. Chem. Hoppe-Seyler*. **376**:71-80.
- Cheng, T. C. and Rodrick, G. E. 1975. Lysosomal and other enzymes in the hemolymph of *Crassostrea virginica* and *Mercenaria mercenaria*. *Comp. Biochem. Physiol.* **52B**: 443-447.
- Chintala, M. M., Ford, S. E., Fisher, W. S., and Ashton-Alcox, K. A. 1994. Oyster serum agglutinins and resistance to protozoan parasites. *J. Shellfish Res.* **13**:115-121.
- Chu, F. L. 1996. Laboratory investigations of susceptibility, infectivity and transmission of *Perkinsus marinus* in oysters. *J. Shellfish Res.* **15**:57-66.
- Chu, F. L. and La Peyre, J. F. 1989. Effect of environmental factors and parasitism on hemolymph lysozyme and protein of American oysters (*Crassostrea virginica*). *J. Invertebr. Path.* **54**:224-232.
- Donovan, M. A. And Laue, T. M. 1991. A novel trypsin inhibitor from the hemolymph of the horseshoe crab, *Limulus polyphemus*. *J. Biol. Chem.* **266**(4):2121-2125.
- Ellis, A. 1987. Inhibition of the *Aeromonas salmonicida* extracellular protease by α_2 -macroglobulin in the serum of rainbow trout. *Microbial Pathogenesis.* **3**:167-177.
- Faisal, M., MacIntyre, E. A., Adham, K. G., Tall, B. D., Kothary, M. H., and La Peyre, J. F. Evidence for the presence of protease inhibitors in eastern (*Crassostrea virginica*) and Pacific (*Crassostrea gigas*) oysters. *Comp. Biochem. Physiol.* **121B**:161-168.

- Garreis, K. A, La Peyre, J. F, and Faisal, M. 1996. The effects of *Perkinsus marinus* extracellular products and purified proteases on oyster defence parameters *in vitro*. *Fish Shellfish Immunol.* 6:581-597.
- Gilardi, M. L. 1996. Transfer of protein/peptides from SDS-PAGE to PVDF or similar inert nylon membranes. SOP # GENPROC 00003. Commonwealth Biotechnologies, Inc. Richmond, VA 23219.
- Harlow, E. and Lane, D. 1988. Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
- Kraut, J. 1977. Serine proteases: Structure and mechanism of catalysis. *Ann. Rev. Biochem.* 46:331-358.
- La Peyre, J. F. 1993. Studies on the oyster pathogen *Perkinsus marinus* (Apicomplexa): Interactions with host defenses of *Crassostrea virginica* and *Crassostrea gigas* and *in vitro* propagation. Ph.D. dissertation, College of William and Mary, VA. pp. 67-68.
- La Peyre, J. F., Faisal, M., and Burrenson, E. M. 1993. *In vitro* propagation of the protozoan *Perkinsus marinus*, a pathogen of the eastern oyster, *Crassostrea virginica*. *J. Euk. Microbiol.* 40:305-310.
- La Peyre, J. F. and Faisal, M. 1995. *Perkinsus marinus* produces extracellular proteolytic factor(s). *Bull. Eur. Assoc. Fish Pathol.* 15:1-4.
- La Peyre, J. F., Schafhauser, D. Y., Rizkalla, E. H., and Faisal, M. 1995. Production of serine proteases by the oyster pathogen *Perkinsus marinus* (Apicomplexa) *in vitro*. *J. Euk. Microbiol.* 42:544-551.

- La Peyre, J. F., Yarnall, H. A., and Faisal, M. 1996. Contribution of *Perkinsus marinus* extracellular products in the infection of eastern oysters (*Crassostrea virginica*). *J. Invertebr. Path.* **68**:312-313.
- Li, M. F. and Fleming, C. 1967. Hemagglutinins from oyster hemolymph. *Can. Jour. Zool.* **45**:1225-1233.
- McKerrow, J. H. 1989. Parasite proteases. *Exp. Parasitol.* **68**:111-115.
- McKerrow, J. H., E. Sun, P. J. Rosenthal, and J. Bouvier. 1993. The Proteases and pathogenicity of parasitic protozoa. *Annu. Rev. Microbiol.* **47**: 821-853.
- Meyers, J. A., Burrenson, E. M., Barber, B. J., and Mann, R. 1991. Susceptibility of diploid and triploid pacific oysters, *Crassostrea gigas*, and eastern oysters, *Crassostrea virginica*, to *Perkinsus marinus*. *J. Shellfish Res.* **10**:433-437.
- Paynter, K. T. 1996. The effects of *Perkinsus marinus* infection on physiological processes in the eastern oyster, *Crassostrea virginica*. *J. Shellfish Res.* **15**:119-125.
- Shumway, S. E. 1996. Chapter 13: Natural Environmental Factors. In "The Eastern Oyster, *Crassostrea virginica*" (V. S. Kennedy, R. I. E. Newell, and A. F. Eble, Eds.), pp.467-513. Maryland Sea Grant College, College Park, Maryland.
- Sousa, M., Moradas-Ferreira, P., and Azevedo, C. 1992. Presence of a trypsin-like protease in starfish sperm acrosome. *J. Exp. Zool.* **261**(3):349-354.
- Thorgersen, I. B., Salvesan, G., Brucato, F. H., Pizzo, S. V., and Enghlid, J. J. 1992. Purification and characterization of and alpha-macroglobulin proteinase inhibitor from the mollusc *Octopus vulgaris*. *Biochem. J.* **285** (Part 2):521-527.

Vasta, G. R., Cheng, T. C., and Marchalonis, J. J. 1984. A lectin on the hemocyte membrane of the oyster (*Crassostrea virginica*). *Cell. Immunol.* **99**:475-488.

Wiedow, O., Harder, J., Bartels, J., Streit, V., and Christophers, E. 1998.

Antileukoprotease in human skin: An antibiotic peptide constitutively produced by keratinocytes. *Biochem. Biophys. Res. Comm.* **248**:904-909.

Table 1. N-terminal amino acid sequence of the p35 target from the eastern oyster.

H₂N – Tyr – Pro – Leu – Glu – His – Asn – Asn – Tyr – Gln – Asp

Table 2. Amino acid analysis of 35 kDa plasma protein target (p35) from *C. virginica*. Results indicate abundance of glycine, alanine, serine, and valine in p35.

	nmol detected	tot nmol in sample	equiv ug	Res/mol
As(x)	0.0231	1.848	0.21252	20.71733
Gl(x)	0.018	1.44	0.18576	16.14337
Ser	0.0397	3.176	0.276312	35.60511
His	0.0059	0.472	0.064664	5.291439
Gly	0.1307	10.456	0.595992	117.2188
Thr	0.0113	0.904	0.091304	10.13445
Ala	0.0881	7.048	0.500408	79.01285
Arg	0.015	1.2	0.1884	13.45281
Tyr	0.0156	1.248	0.203424	13.99092
Cystine	0	0	0	0
Val	0.0367	2.936	0.290664	32.91455
Met	0.003	0.24	0.03144	2.690562
Trp	0	0	0	0
Phe	0.0107	0.856	0.125832	9.596339
Ile	0.0066	0.528	0.059664	5.919237
Leu	0.0103	0.824	0.093112	9.237597
Lys	0.0025	0.2	0.0256	2.242135
Pro	0.0228	1.824	0.176928	20.44827
HyPro	0	0	0	0
EACA	0	0	0	0
B-Ala	0	0	0	0
Cys	0	0	0	0

MGS IN SAMPLE: 0.003122

Figure Legends

Figure 1. Comparison of diluents on *P. marinus* protease digestions. The products of *P. marinus* protease digestions of *C. virginica* plasma proteins in the presence of PBS (a, b) and plasma-based diluents (c, d) were analyzed by 12% reducing SDS-PAGE. Each gel was immunoblotted (a, c) and colloidal gold stained (b, d) to reveal the total protein.

Lane 1, biotinylated molecular weight markers; lane 2, 1.0 μ g ECP only; lane 3, 1.0 μ g ECP and 1.0 μ g *C. virginica* proteins; lane 4, 1.0 μ g *C. virginica* proteins only. The arrows in 1a, 1b, 1c, and 1d indicate the 35 kDa plasma protein target in *C. virginica*.

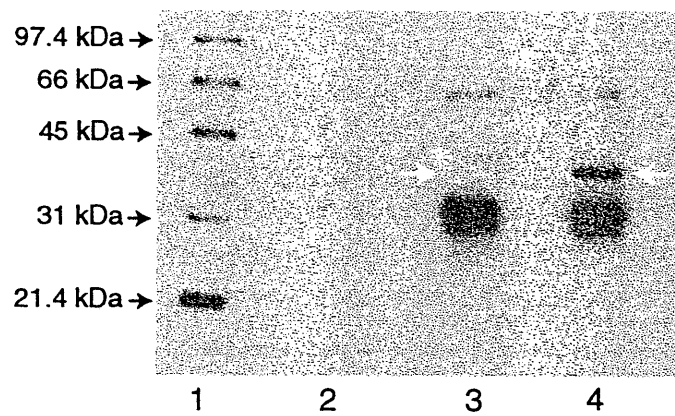
Asterisks in 1a, 1c, and 1d denote possible degradation products of *P. marinus* ECP digestion.

Figure 2. Comparison of diluents on *P. marinus* protease digestions. The products of *P. marinus* protease digestions of *C. gigas* plasma proteins in the presence of PBS (a, b) and plasma-based diluent (c, d) were analyzed by 12% reducing SDS-PAGE. Each gel was immunoblotted (a, c) and colloidal gold stained (b, d) to reveal total protein. Lane 1, biotinylated molecular weight markers; lane 2, 1.0 μ g ECP only; lane 3, 1.0 μ g ECP and 1.0 μ g *C. gigas* proteins; lane 4, 1.0 μ g *C. gigas* proteins only. The circles in 2a, 2b, 2c, and 2d denote partial digestion of *C. gigas* plasma proteins by *P. marinus* ECP. Brackets in 2a, 2b, 2c, and 2d denote possible degradation products of *C. gigas* plasma proteins by *P. marinus* ECP.

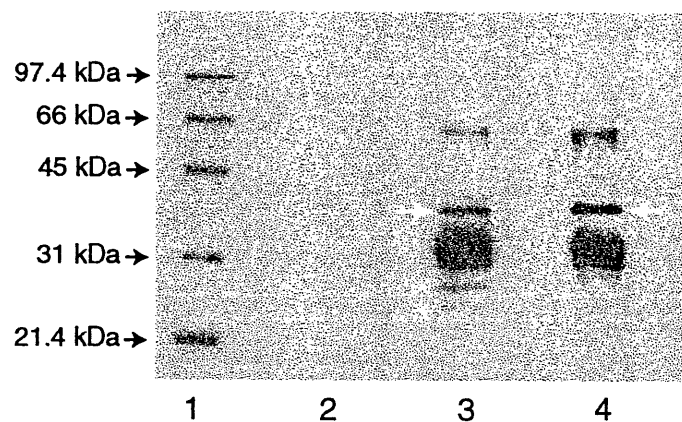
Figure 3. Comparison of diluents on α -chymotrypsin digestion of *C. virginica* plasma proteins. The products of α -chymotrypsin digestions of *C. virginica* plasma proteins in the presence of PBS (a, b) and plasma-based diluent (c, d) were analyzed by 12% reducing SDS-PAGE. Each gel was immunoblotted (a, c) to reveal the plasma proteins and colloidal gold stained (b, d) to reveal the total protein. Lane 1, biotinylated molecular weight markers; lane 2, 1.0 μ g α -chymotrypsin only; lane 3 1.0 μ g α -chymotrypsin and 1.0 μ g *C. virginica* plasma proteins; lane 4, 1.0 μ g *C. virginica* plasma proteins only. The arrows in 3a, 3b, 3c, and 3d indicate the 35 kDa plasma protein target in *C. virginica*. Asterisks in 3a, 3b, and 3c denote possible degradation products of α -chymotrypsin digestion.

Figure 4. Two-dimensional electrophoretic analysis of *C. virginica* plasma proteins. Undigested (a, b) and *P. marinus* protease-digested (c, d) *C. virginica* plasma protein samples were analyzed by two-dimensional electrophoretic analysis (isoelectric focusing – first dimension, 12% reducing SDS-PAGE, second dimension). Each gel was immunoblotted (a, c) and colloidal gold stained (b, d) to reveal the total protein. Lane 1, biotinylated molecular weight markers; 2D gel, 1.0 μ g *C. virginica* plasma proteins; lane 2, 1.0 μ g *C. virginica* plasma proteins. The circle with an arrow in 4a, 4b, 4c, and 4d denotes the 35 kDa plasma protein target in *C. virginica*. The circle with an asterisk in 4a and 4c denotes a possible additional plasma protein target.

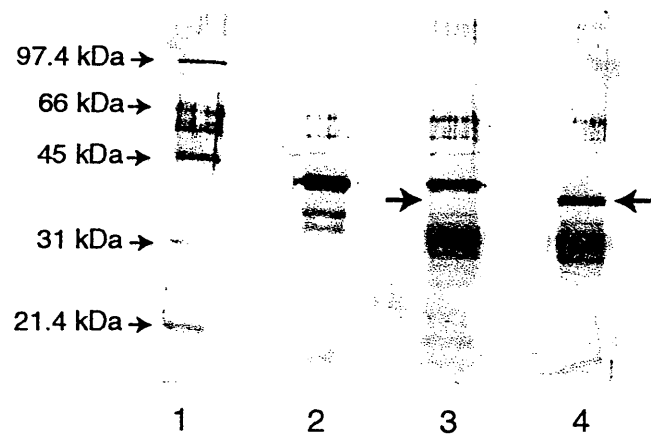
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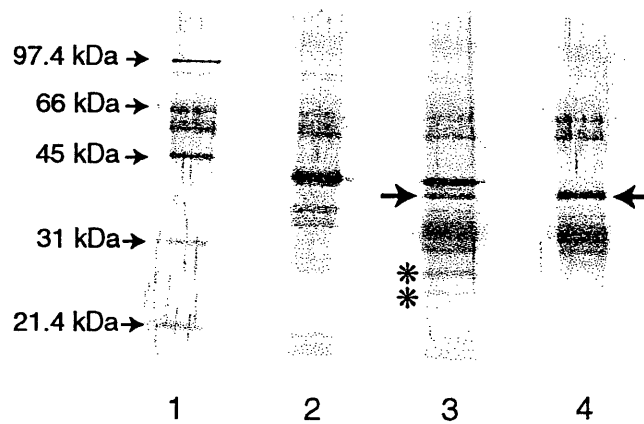
1c.



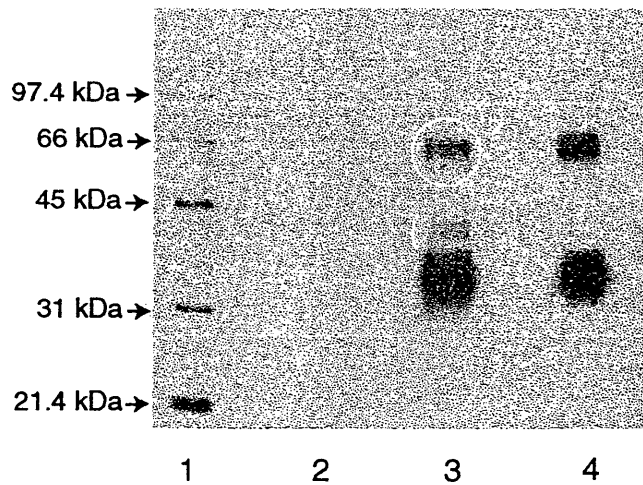
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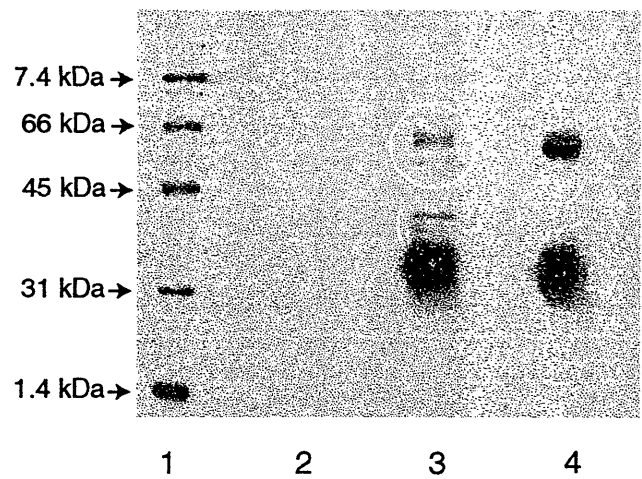
1d.



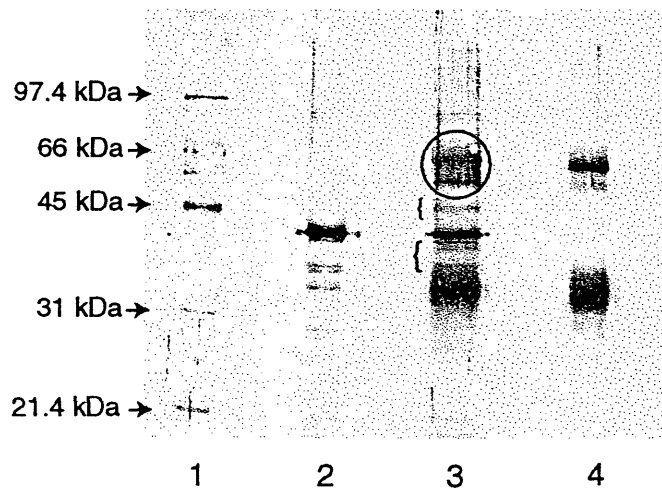
2a.



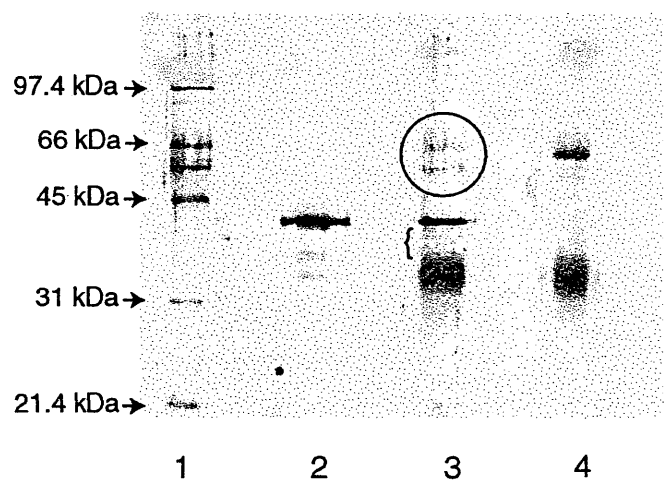
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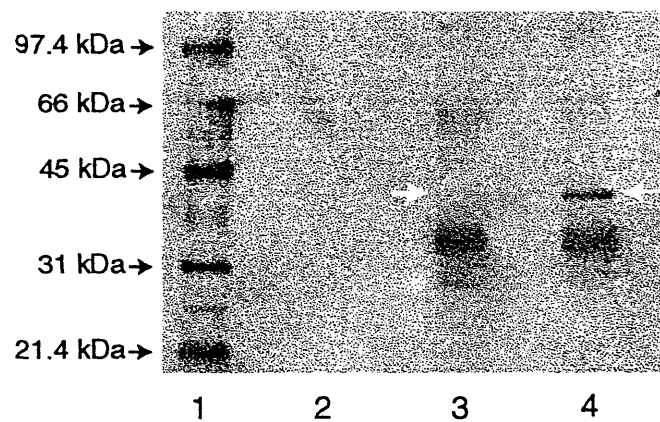
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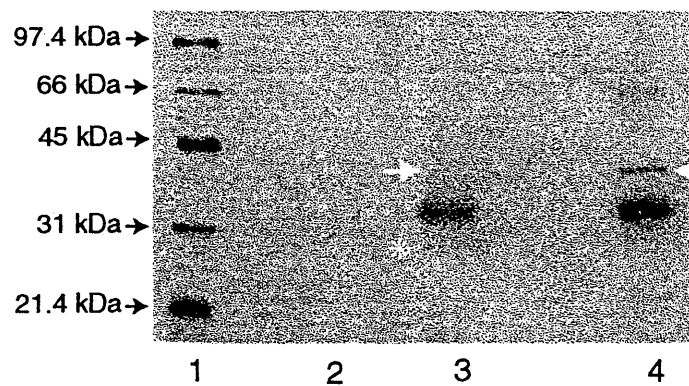
2d.



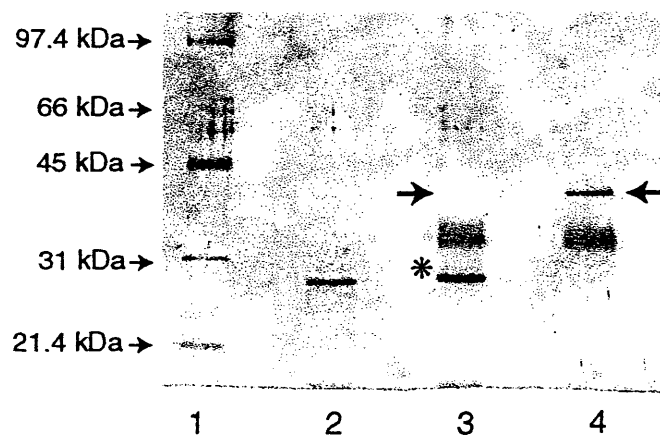
3a.



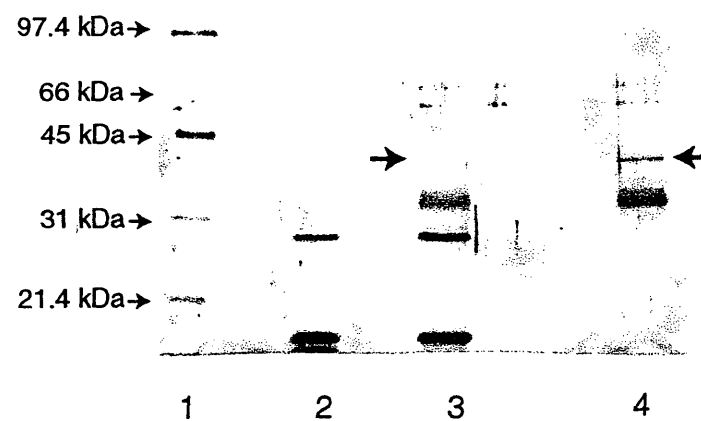
3c.



3b.

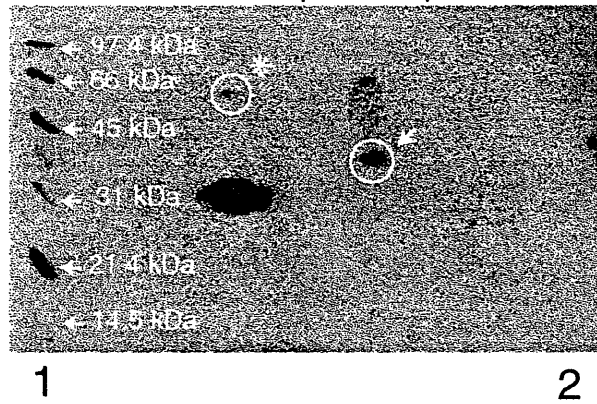


3d.

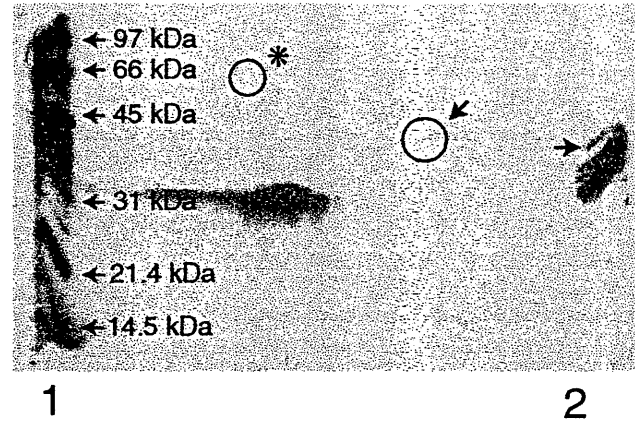


4a.

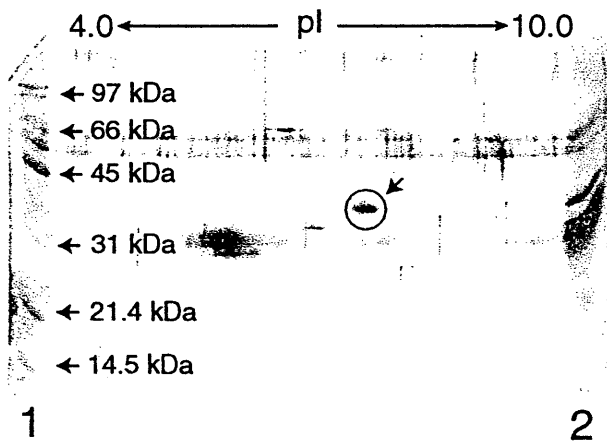
pl Range
5.9-6.6



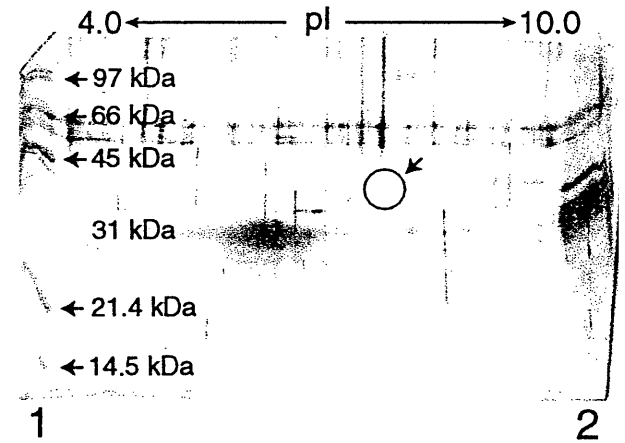
4c.



4b.



4d.



Chapter 2

Protease inhibitory activity in selectively bred families of eastern oysters, *Crassostrea virginica*

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Abstract

Selected stocks of eastern oysters, *Crassostrea virginica*, were interbred to produce ten families with high inter- and intra-family genetic variability. In an effort to identify potential biochemical markers for resistance to Dermo, a disease afflicting eastern oysters, subsamples of two year old oysters from each of these families were assessed for protease inhibitory activity (PI) against proteases of *Perkinsus marinus*, the causative agent of Dermo disease. Prevalence and intensity of the pathogen were assessed via Ray's Fluid Thioglycollate Medium (RFTM) test. Family survival under field challenge, disease intensity, and protease inhibitory activity were compared to evaluate family performance. In the laboratory, three families had the highest survival, lowest average number of parasite cells, and highest average PI. In field challenges, the same three families exhibited the lowest cumulative mortality. Among all families, disease intensity was negatively correlated with protease inhibitory activity. Mortality patterns also indicated an inverse relationship between disease intensity and protease inhibitory activity.

Introduction

Perkinsus marinus is a protozoan pathogen of the eastern oyster, *Crassostrea virginica*, and is the causative agent of the disease known as Dermo (Andrews, 1988). This pathogen has had a severe economic impact on the commercial oyster fishery in the Chesapeake Bay. Commercial landings have fallen from 3 million bushels per year in 1960 to less than 100,000 bushels per year in 1994 (Bureson and Ragone Calvo, 1996). One approach taken toward the rebuilding of oyster populations in endemic disease environments has been the selective breeding of disease resistant oysters (Haskin and Ford, 1979; Ewart et al., 1988; Matthiessen et al., 1990; Matthiessen and Davis, 1991; Bureson, 1991). Groups of oysters that have exhibited low mortality under heavy disease conditions have been selected for interbreeding to produce potentially disease resistant lines. This type of selective breeding has had considerable success with respect to MSX (*Haplosporidium nelsoni*) over the past two decades (Gaffney and Bushek, 1996); however, the success in generating Dermo-resistant oysters has been limited (Gaffney and Bushek, 1996).

Selecting oysters for disease resistance is not a complex process and, at present, it is thus far the most realistic approach for producing stocks with low mortality from MSX and Dermo. Individual oysters that survive and grow well in the face of natural field challenges are selected as spawners for the next generation; their progeny are likewise field-tested to provide parents for the subsequent generation, and this process is

continued (Gaffney and Bushek, 1996). Despite its technical ease, this approach possesses several drawbacks. For example, inter-annual variation in uncontrollable abiotic factors, such as temperature and salinity, can dramatically affect disease intensity (Gaffney and Bushek, 1996). Therefore, such factors can easily confound one's ability to distinguish between high survival due to resistance and high survival due to low disease intensity. The slow growth of oysters and the chronic nature of Dermo can also make survival a fairly cumbersome selection parameter. It may take several years to determine whether a developing stock is resistant to the disease (Gaffney and Bushek, 1996). Alternatively, physiological or biochemical disease resistance markers that can be examined early in oyster development are more attractive alternatives. Furthermore, identifying such markers for disease resistance will contribute to understanding the biochemical aspects of *P. marinus* molecular pathogenesis. However, presently, there are no known genetic or biochemical markers for Dermo disease resistance in the eastern oyster.

One class of potential disease resistance markers could be those molecules involved in neutralizing the virulence factors of *P. marinus*. In this vein, La Peyre and Faisal (1995) originally hypothesized that extracellular proteins (ECP) and serine proteases secreted by *P. marinus* might be important virulence factors. Evidence to support this hypothesis has been garnered over recent years (La Peyre et al., 1996; Garreis et al., 1996; Faisal et al., 1998). Previous work in our laboratory demonstrated that eastern oysters possess low molecular weight inhibitory activity directed against these *P. marinus* proteases. Specifically, low molecular weight plasma molecules (8 - 10 kDa) were observed to inhibit these serine proteases from digesting a specific host plasma protein *in*

vitro. Based on this observation, we hypothesized that the observed anti-proteolytic activity in eastern oysters might be an important host defense factor. Thus, studies have been initiated to determine if these anti-proteases can serve as biochemical markers for Dermo resistance.

To test whether there is a relationship between protease inhibitory activity and disease resistance, ten *C. virginica* families possessing different genetic backgrounds were examined. We hypothesized that the genetic variability would facilitate the identification of phenotypically distinct groups in terms of their ability to resist infections with *P. marinus*. Furthermore, we hypothesized that such a phenotypic distinction would be correlated with protease inhibitory activity.

Materials and Methods

Oysters

Oyster stocks of specific parentage were bred at the Haskin Shellfish Research Laboratory (HSRL) (Rutgers University, Cape Shore, NJ) in 1996. The male and female parent for each family is listed in Table 1. Oysters from each group were individually examined and sexed microscopically. Crosses were 1 x 1 (male from one group crossed with a female from another group). Females were stripped and eggs cleaned by consecutive filtration over a 60 μ m and 15 μ m screen. Following filtration, eggs were transferred to 100 ml of seawater and counted in duplicate. Eggs were checked for the presence of sperm that would indicate hermaphroditism. Samples indicating hermaphroditism were discarded and a new female was used. Males were stripped and sperm activity was qualitatively assessed. The amount of sperm used for fertilization was

determined by the activity and density of the sperm from each male. Eggs were fertilized in a small plastic container (500 ml) by adding sperm at a ratio of 5:1, sperm to egg. The fertilization rate was estimated one hour post-fertilization by the following equation: (number of fertilized eggs/total number of observed eggs) X 100. Each family was then labeled with the appropriate designation (see Table 1). Following fertilization, larvae were reared in aerated 211-liter tanks at a temperature of 25-27°C and 17-26 ppt salinity. The food mixture consisted of *Isochrysis galbana* and *Chaetoceros calcitrans* at a density of 60,000 – 100,000 cells/ml. Water changes were made every other day by siphoning out water with a hose and filtering larvae onto mesh screens. Competent larvae were set cultchlessly using a 10^{-4} M epinephrine solution according to the methods adapted from Coon et al. (1986). Set larvae were transferred to 211 liter downwelling tanks for 10-14 days or until spat reached a shell length of approximately 1.0 mm. Spat were transferred to upwelling raceways and maintained until shell length reached 10.0 mm. Juvenile oysters were transferred to 1/8-inch mesh bags, then rotated to intertidal grow out racks in Delaware Bay. Oysters were maintained at Cape Shore, NJ for two years until the initiation of disease challenges. All oysters were exposed to ambient Delaware Bay water during their maintenance at HSRL.

In June 1998, oysters were transferred to the Virginia Institute of Marine Science and divided into a laboratory group and a field group. The laboratory group comprised subsamples of 50 oysters from each family. Laboratory oysters were acclimated in filtered York River water (25° C, 18 ppt) in a 156-gallon tank for one week. All families were sampled for Dermo and MSX diagnosis and were subsequently maintained in York River water filtered through two 1 µm polypropylene filters (Filter Equipment Co., Inc.,

Wall, NJ). Oysters were held in ambient filtered York River water for the duration of the experiment. Oysters were fed 0.3 g algae paste/oyster per day (*Thalassosira weissflogii*) and water was changed every other day. The remaining oysters constituted the field group and were placed in sites in the Chesapeake Bay thought to be endemic only for Dermo to allow for natural challenges to *P. marinus*. Oysters were sampled in October and December 1998, and March 1999 for determination of growth (shell height) and survival.

Hemolymph

In June 1998, twenty oysters were taken from each family and notched using a circular saw to expose a small opening into the mantle cavity. Oysters were returned to the tanks and held for another 3 days to recuperate from notching. Oysters were bled from the adductor muscle using a 5 cc syringe and 25 gauge needle. Hemolymph was centrifuged at 400 x g for 10 minutes at 4°C. The cell-free supernatant was filtered through a 0.22 µm syringe-top filter (Costar Scientific Corp., Cambridge, MA). The resulting plasma was centrifuged in a Centricon-10 apparatus (Amicon Inc., Beverly, MA) to fractionate low molecular weight plasma (<10 kDa). The filtrate containing less than 10 kDa molecules was stored at -20°C until further use. Eight weeks later the procedure was repeated with the remaining oysters from each family.

Disease Diagnosis

After obtaining hemolymph from oysters from each family, oysters were assayed for

P. marinus and *H. nelsoni* infections. Oysters were shucked and gill, mantle, and rectal tissues were sampled for presence of *P. marinus*. Diagnosis was performed using Ray's Fluid Thioglycollate Method (RFTM) (Ray, 1952, 1966) and infections were scored according to the method described by Mackin (1962). For MSX diagnosis, two oysters from each family were shucked and oyster tissues were prepared for histological examination using the methods described by Stokes et al. (1995).

Protease Inhibitory Activity

Protease inhibitory activity within oysters was detected and quantified using the Hide Powder Azure as a substrate as detailed by Bender et al. (1992). The protocol was modified for microtiter plate assays. First, *P. marinus* extracellular proteins (ECP) containing serine proteases were derived from culture supernatants as described by La Peyre et al. (1995). A standard curve of *P. marinus* ECP was generated by assessing undiluted *P. marinus* protease and two-fold dilutions (i.e., 1:4, 1:8, 1:16, 1:32, 1:64, and 1:128) in a 96 well microtiter plate (Costar). All dilutions employed in the standard curve were made using cold (4°C) artificial seawater (Forty Fathoms Marine Mix Artificial Sea Water, 22 g/L) and were kept on ice. Four microliters of each dilution from the standard curve was added to wells in triplicate followed by 50 µl of room temperature (RT) artificial sea water. Fifty-four microliters of artificial seawater at RT was added as a blank. Four microliters of undiluted protease was added to separate wells in triplicate followed by 50 µl of low molecular weight (LMW) oyster plasma containing PI activity. Negative controls consisted of 54 µl of plasma only. A protease control consisted of 4 µl undiluted protease plus 50 µl RT artificial seawater. The blank for the protease control

consisted of 54 μ l of RT artificial seawater only. Mixtures of protease and plasma were incubated at RT for 15 minutes on a shaker. Following incubation, 100 μ l of substrate suspension (Hide Powder Azure, 0.02 g/ml, 150 mM Tris, 30 mM CaCl_2 , 0.05% Brij, 20% sucrose, pH 7.5) was added to each well. Plates were incubated at 37°C on a shaker for 3 hours. After incubation, 100 μ l of cold artificial seawater was added to each well to stop the reaction. Plates were centrifuged at 2700 x g for 15 minutes at 4°C. One hundred microliters of supernatant were removed from each well and transferred to another 96 well microtiter plate. Plates were read at 540 nm on a Titertek® Multiskan® MCC/340 plate reader (Labsystems, Finland) using DeltaSoft 3 software (BioMetallics, Inc., Princeton, NJ). Based on the protease standard curve, reduction in A_{540} due to the inhibition by LMW *C. virginica* plasma was calculated and expressed as ng *P. marinus* protease inhibited/50 μ l LMW plasma.

Statistical Analysis

For oysters held in the laboratory, a one-way analysis of variance (ANOVA) was performed among all families with respect to Mackin Scale and PI. The statistical analyses were performed using Minitab (version 12.1, Minitab, Inc., State College, PA). The RFTM scoring for individual oysters from June and August 1998, among all families, were plotted on the Mackin Scale (negative, rare, light, light to moderate, moderate, and heavy) and compared by one-way ANOVA (Minitab) with respect to PI. Oysters that were scored with moderate to heavy infections (n=4) were combined with oysters that were scored with heavy infections. A Tukey's multiple comparisons test was performed according to the methods described by Zar (1996) to detect differences in PI

between ratings on the Mackin scale. Sigma Plot (version 4.01, SPSS, Inc., San Rafael, CA) was used to perform a simple linear regression to correlate Mackin scale with PI.

Results

MSX and Dermo Diagnosis

Overall, there was a 21% prevalence of *H. nelsoni* and an 87% prevalence of *P. marinus* at the start of the experiment among all families (data not shown). The degree of intra-family *H. nelsoni* prevalence was not determined. However, there was a wide range of *P. marinus* prevalence between families. Seven families exhibited greater than 94% prevalence while the remaining three families exhibited 40%, 70%, and 80% prevalence, respectively (Table 2). Furthermore, the intensity of *P. marinus* infections among families varied from rare to moderate infections.

Inter-family Disease Intensity, PI, and Survival

In families kept in the laboratory, four families showed above 70% survival from June through August (Table 3). Families PG-14, PG-2, PG-6, and PG-1 had the highest survival (100%, 89%, 85%, and 71% respectively). All other families had less than 50% survival with PG-21, PG-24, and PG-10 having survival less than 30%. In the field exposures, the same families exhibited the highest survival (Table 3). The six remaining families demonstrated survival consistently below 40%. PG-21 appeared to be the most susceptible, exhibiting a survival of only 3%. In all families, with the exception to PG-24 and PG-10, laboratory-held animals exhibited higher percent survival than their counterparts in the field.

Families PG-14, PG-1, PG-2, and PG-24 maintained the lightest infections of *P. marinus* from June through August (Figure 1a). Also, PG-14 and PG-2 had the highest mean PI of all families (1177 and 1302 ng ECP inhibited, respectively), although PG-1 had the fourth highest mean PI (956 ng ECP inhibited) (Figure 1b). PG-24 had the lowest PI at 204 ng ECP inhibited. Families that appeared to be most susceptible to *P. marinus* infections were PG-20, PG-10, and PG-26 (Figure 1a). Overall, the inverse correlation among families between infection level (median Mackin score) and protease inhibitory activity (median PI) was suggestive but not statistically significant ($r = -0.41$, $p = 0.24$).

To demonstrate more clearly the differences between the families, family-specific PI values were plotted against survival in the laboratory (Figure 2). The correlation between family mean PI and survival was significant ($r = 0.685$, $p = 0.029$). Interestingly, PG-10 exhibited high PI, yet had low survival in the laboratory and in the field. Conversely, PG-6 exhibited high survival, yet had only moderate PI.

Relationship of PI to Disease Intensity

To correlate PI with Mackin scale, individual oysters, irrespective of family, were plotted with their Mackin scale scorings versus their PI (Figure 3). Regression of PI against Mackin scale indicated a negative correlation ($r = -0.31$). The relationship between Mackin scale and PI was weak ($r^2 = 0.096$), however, the result was still highly significant ($p < 0.0005$). One-way ANOVA and a Tukey's multiple comparisons test detected a significant difference in PI between negatively and heavily infected oysters ($p < 0.005$ and $p = 0.005$, respectively).

Discussion

There appear to be distinct differences between the selectively bred oyster families with regard to PI and susceptibility to *P. marinus* infections. Preliminary diagnosis of *P. marinus* infections revealed differences in prevalence and intensity between families. Some families such as PG-14, PG-1, and PG-2 had rare infections prior to the experiment (Table 2) and maintained either rare or light infections throughout the study. Conversely, families PG-20, PG-10, and PG-26 were diagnosed with light or moderate infections prior to the experiment (Table 2) and infections intensified to moderate and moderate/heavy. It is interesting to note that some MSX-resistant parental stocks were used to generate the families, however, low *P. marinus* susceptibility was observed in some of these families. Thus, although the occurrence of moderately and heavily Dermo infected families that have MSX-resistant parents is consistent with the findings by Chintala and Fisher (1989) and Burreson (1991), such parents can yield seemingly Dermo-resistant progeny. A difference between laboratory and field survival in the families was also detected (Table 3), however, this would be expected. The lower survival of families in the field may have been reflective of the sustained challenge over a longer time period to *P. marinus* in the field or exposure to other pathogens.

Three of the four families that exhibited low disease intensity, PG-14, PG-1, and PG-2 also exhibited high PI. Two of the three families with the highest disease intensity, PG-20 and PG-26, also exhibited low PI (Figure 1a and 1b). It is possible that elevated PI

played an important role in suppressing *P. marinus* infections in PG-14, PG-1, and PG-2 by inhibiting pathogen proteases while the lower PI in PG-20 and PG-26 resulted in higher susceptibility. Although no definitive cause and effect relationship can be concluded from this association, it is suggestive that either PI is responsible for limiting or preventing *P. marinus* infections, or protease inhibitors are the targets of *P. marinus* infections. Alternatively, it is possible that under heavy infection pathogen proteases deplete the host repertoire of inhibitors by forming protease-inhibitor complexes. It is possible that under heavy *P. marinus* infections, the pathogen may destroy or suppress an oyster's ability to produce inhibitors. In a study by Ford (1986), a dramatic decline in hemolymph protein concentration in MSX-susceptible oysters was observed with a concurrent increase in MSX intensity. Ford suggested that such a depression in hemolymph proteins was due to long-term systemic parasitism and that their loss during *H. nelsoni* infections reflected their overall importance to the health of the oyster.

Because of the low prevalence of *H. nelsoni* in preliminary diagnoses, it cannot be precluded that MSX may play a minor role in affecting the PI levels observed in the experiment. However, such a proviso would have to be made for all possible pathogens and commensals, even under the most stringent of culture conditions.

The production of phenotypically variable families of oysters enabled us to determine if a relationship existed between PI and disease intensity. When average family Mackin scale values were compared to their respective PI levels, an inverse correlation was observed (Figure 1a and 1b). This correlation, however, was only suggestive and was not statistically significant. One factor that might have confounded a significant relationship might have been previous exposure to other pathogens.

This correlation, albeit not statistically significant, prompted us to explore the relationship between PI and disease intensity, irrespective of family. Box plots of individual oysters, grouped according to their Mackin scale scoring and plotted versus its PI, showed a negative correlation between the two (Figure 3). Furthermore, one-way ANOVA and multiple comparison tests revealed a significant difference in PI between the negatively and heavily infected oysters. The negative correlation between disease intensity and PI, as well as the difference between the negative and heavy infections, further supports the hypothesis that PI may be an important defense factor in fighting *P. marinus* infections.

Other studies have attempted to identify biochemical and cellular correlates of disease resistance in *C. virginica*. In a study by Ford (1986), hemolymph proteins from MSX-resistant and susceptible *C. virginica* were compared after exposure to *H. nelsoni*. Ford attempted to correlate disease resistance with changes in serum protein by comparing electrophoretic profiles of oysters from both groups. Ford observed variability in the electrophoretic profiles with no definitive differences in banding patterns between the resistant and susceptible groups being discerned. Chu and La Peyre (1993) compared three separate stocks of oysters to determine differences in specific humoral and cellular responses to *P. marinus* challenge. They observed the lowest cumulative mortality and the highest serum protein and lysozyme concentration in the Deep Water Shoals (DWS) stock (James River, VA). Also, the percentage of granulocytes in the DWS stock was greater than that in the other groups. Chu and La Peyre speculated that this difference might have provided the DWS stock with a physiological advantage over the other groups, rendering them less susceptible to

infection. In another study, Ling (1990) observed an increase in hemocyte numbers (hemocytosis) in MSX-resistant oysters compared to MSX-susceptible oysters when challenged with *H. nelsoni*.

Protease inhibitors have been identified in a number of molluscan species (Armstrong and Quigley, 1992), however, they have not been implicated in disease resistance in marine molluscs. In a study by Fryer et al. (1991), resistant strains of the snail, *Biomphalaria glabrata*, exhibited significantly higher PI to the trematode, *Schistosoma mansoni*, than did susceptible strains. The endogenous PI may not necessarily be responsible for resistance in strains of *B. glabrata* since the PI was assessed by its activity against commercial proteases and not proteases of *S. mansoni*. Teleost protease inhibitors have also been suggested as important mediators of disease resistance. In a study by Ellis (1991), an α_2 -macroglobulin-like molecule was purified from rainbow trout (*Oncorhynchus mykiss*) sera and its neutralizing activity demonstrated against a protease of *Aeromonas salmonicida*, a major virulence factor of the pathogen. Freedman (1991) compared the α_2 -macroglobulin-like neutralizing activity in rainbow trout and brook trout (*Salvelinus fontinalis*) to *A. salmonicida* and found that brook trout, which are more susceptible to furunculosis, exhibited lower activity.

Despite the biochemical or cellular correlations drawn by previous investigators, the need to identify definitive markers of resistance still remains. Our results demonstrate that among genetically diverse and phenotypically distinct families of oysters, differential resistance to *P. marinus* infections can be correlated with levels of endogenous protease inhibitors. This association suggests that PI may serve as an indicator or marker of disease resistance.

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References

- Armstrong, P. B. and Quigley, J. P. 1992. Humoral immunity: α_2 -macroglobulin activity in the plasma of mollusks. *Veliger*. **35**:161-164.
- Andrews, J. D. 1988. Epizootiology of the disease caused by the oyster pathogen *Perkinsus marinus* and its effect on the oyster industry. *Am. Fish. Soc. Spec. Publ.* **18**:47-63.
- Bender, R. C., Fryer, S. E., and Bayne, C. J. 1992. Proteinase inhibitory activity in the plasma of a mollusc: Evidence for the presence of α -macroglobulin in *Biomphalaria glabrata*. *Comp. Biochem. Physiol.* **102B**(4):821-824.
- Burreson, E. 1991. Effects of *Perkinsus marinus* infection in the eastern oysters, *Crassostrea virginica* (Gmelin, 1791): I. Susceptibility of native and MSX-resistant stocks. *J. Shellfish Res.* **10**:417-423.
- Burreson, E. M. and Ragone Calvo, L. M. 1996. Epizootiology of *Perkinsus marinus* disease of oysters in Chesapeake Bay, with emphasis on data since 1985. *J. Shellfish Res.* **15**:17-34.
- Chintala, M. M. and Fisher, W. S. 1989. Comparison of oyster defense mechanisms for MSX-resistant and -susceptible stocks held in Chesapeake Bay. *J. Shellfish Res.* **8**:846-847.
- Chu, F.-L. E. and La Peyre, J. F. 1993. Development of disease caused by the parasite, *Perkinsus marinus*, and defense-related hemolymph factors in three populations of oysters from the Chesapeake Bay, USA. *J. Shellfish Res.* **12**(1):21-27.
- Coon, S. L., Bonard, D. B., and Weiner, R. M. 1986. Chemical production of cultchless spat using epinephrine and norepinephrine. *Aquaculture*. **58**:255-262.

- Ellis, A. E. 1991. Inhibition of the *Aeromonas salmonicida* extracellular protease by α_2 -macroglobulin in the serum of rainbow trout. *J. Fish Dis.* **14**:265-277.
- Ewart, J. W., Cole, R., and Tinsman, J. 1988. Growth and survival of hatchery produced MSX resistant oyster stocks in Delaware Bay. *J. Shellfish Res.* **7**:543.
- Faisal, M., MacIntyre, E. A., Adham, K. G., Tall, B. D., Kothary, M. H., and La Peyre, J. F. 1998. Evidence for the presence of protease inhibitors in eastern (*Crassostrea virginica*) and Pacific (*Crassostrea gigas*) oysters. *Comp. Biochem. Physiol.* **121B**:161-168.
- Ford, S. E. 1986. Comparison of hemolymph proteins from resistant and susceptible oysters, *Crassostrea virginica*, exposed to the parasite *Haplosporidium nelsoni* (MSX). *J. Invertebr. Path.* **47**:283-294.
- Freedman, S. J. 1991. The role of α_2 -macroglobulin in furunculosis: a comparison of rainbow trout and brook trout. *Comp. Biochem. Physiol.* **98B**:549-553.
- Fryer, S. E., Bender, R. C. and Bayne, C. J. 1991. Proteinase inhibitory activity in the plasma of the gastropod mollusc, *Biomphalaria glabrata*. *Dev. Comp. Immunol.* **15**:S45.
- Gaffney, P. M. and Bushek, D. 1996. Genetic aspects of disease resistance in oysters. *J. Shellfish Res.* **15**(1):135-140.
- Garreis, K. A, La Peyre, J. F, and Faisal, M. 1996. The effects of *Perkinsus marinus* extracellular products and purified proteases on oyster defence parameters *in vitro*. *Fish Shellfish Immunol.* **6**:581-597.
- Haskin, H. H. and Ford, S. E. 1979. Development of resistance to *Minchinia nelsoni*

- (MSX) mortality in laboratory-reared and native oyster stocks in Delaware Bay.
Mar. Fish. Rev. **41**:54-63.
- La Peyre, J. F. and Faisal, M. 1995. *Perkinsus marinus* produces extracellular
 Proteolytic factor(s). *Bull. Eur. Assoc. Fish Pathol.* **15**:1-4.
- La Peyre, J. F., Schafhauser, D. Y., Rizkalla, E. H., and Faisal, M. 1995. Production of
 serine proteases by the oyster pathogen *Perkinsus marinus* (Apicomplexa) *in vitro*. *J.*
Euk. Microbiol. **42**:544-551.
- La Peyre, J. F., Yarnall, H. A., and Faisal, M. 1996. Contribution of *Perkinsus marinus*
 extracellular products in the infection of Eastern oysters (*Crassostrea virginica*). *J.*
Invertebr. Path. **68**:312-313.
- Ling, W.-J. 1990. Cellular and humoral responses of resistant and susceptible oysters,
Crassostrea virginica, to the infection of *Haplosporidium nelsoni* (MSX). M. S.
 Thesis. Univ. Conn. 102 pp.
- Mackin, J. G. 1962. Oyster disease caused by *Dermocystidium marinum* and other
 microorganisms in Louisiana. *Publ. Inst. Marine Sci. Univ. Texas.* **7**:132-229.
- Matthiessen, G. C., Feng, S. Y., and Leibovitz, L. 1990. Patterns of MSX
 (*Haplosporidium nelsoni*) infection and subsequent mortality in resistant and
 susceptible strain of the eastern oyster, *Crassostrea virginica* (Gmelin, 1791), in
 New England. *J. Shellfish Res.* **9**:359-365.
- Matthiessen, G. C. and Davis, J. P. 1991. Growth, disease resistance and survivorship of
 diploid and triploid eastern oysters in an MSX environment. *J. Shellfish Res.*
10:298-299.

- Oliver, J. L., Lewis, T. D., Faisal, M., and Kaattari, S. L. 1999. Analysis of the effects of *Perkinsus marinus* proteases on plasma proteins of the eastern (*Crassostrea virginica*) and Pacific oyster (*Crassostrea gigas*). *J. Invertebr. Path.* **74**:173-183.
- Ray, S. M. 1952. A culture technique for the diagnosis of infection with *Dermocystidium marinum* (Mackin, Owen, and Collier) in oysters. *Science*. **116**:360-361.
- Ray, S.M. 1966. A review of the culture method for detecting *Dermocystidium marinum*, with suggested modifications and precautions. *Proc. Natl. Shellfish Assoc.* **54**:55-69.
- Stokes, N. A., Siddall, M. E., and Bureson, E. M. 1995. Detection of *Haplosporidium nelsoni* (Haplosporidia: Haplosporidiidae) in oysters by PCR amplification. *Dis. Aq. Org.* **23**:145-152.
- Vrijenhoek, R. C., Ford, S. E., and Haskin, H. H. 1990. Maintenance of heterozygosity during selective breeding of oysters for resistance to MSX disease. *J. Hered.* **81**:418-423.
- Zar, J. H. 1996. *Biostatistical analysis*, 3rd edition. Prentice Hall, Englewood Cliffs, New Jersey. 662 pp.

Table 1. Lineages of ten oyster families selected for Dermo resistance. Origins of each parental female and male are listed.

Family	Parental Female	Parental Male
PG-1	F ₇ MSX disease resistant line (Delaware Bay) ¹	F ₁ CROSBreed line ²
PG-2	F ₁ CROSBreed line	F ₇ MSX disease resistant line (Delaware Bay)
PG-6	Maine wild type x F ₇ MSX disease resistant line (Delaware Bay)	Louisiana wild type
PG-10	Maine wild type x F ₇ MSX disease resistant line (Delaware Bay)	Maine stock x F ₇ MSX disease resistant line (Delaware Bay)
PG-14	Maine stock x F ₇ MSX disease resistant line (Delaware Bay)	Texas wild type x Maine wild type
PG-18	Naturalized Oregon stock ³	Alabama wild type x Maine wild type
PG-20	Naturalized Oregon stock	Alabama wild type x Maine wild type
PG-21	Maine wild type	F ₁ CROSBreed line
PG-24	F ₁ CROSBreed line	Maine wild type
PG-26	Maine wild type	F ₆ MSX disease resistant line (Delaware Bay) x F ₅ MSX disease resistant line (Long Island) ⁴

¹ - Vrijenhoek et al., 1990.

² – CROSBreed lines were produced by mating several MSX resistant lines so that any one progeny was a hybrid of two MSX resistant lines as described by Vrijenhoek et al., 1990.

³ – Oysters were imported from the east coast of the U.S. to the west coast and have been kept there for an unspecified number of generations. Their original genetic origin is unknown.

⁴ – Disease resistant lines were produced from either Delaware Bay survivors or from survivors of Long Island stocks (transplanted to Delaware Bay) (Haskin and Ford, 1987).

Table 2. Initial and ending Dermo intensity and prevalence of each crossbreed family.

Family	Mean Initial Dermo Intensity and Prevalence¹	Ending Dermo Intensity and Prevalence²
PG-14	0.575 (40%)	0.97 (100%)
PG-2	0.425 (70%)	2.11 (100%)
PG-6	1.921 (95%)	1.75 (93%)
PG-1	0.550 (80%)	1.39 (100%)
PG-18	2.250 (100%)	1.58 (100%)
PG-26	3.079 (100%)	3.63 (100%)
PG-20	2.083 (100%)	5.00 (100%)
PG-21	2.206 (100%)	1.81 (100%)
PG-24	1.250 (94%)	2.00 (83%)
PG-10	2.025 (100%)	4.60 (100%)

¹*P. marinus* infections measured in June 1998.

²*P. marinus* infections measured in August 1998.

Table 3. Percent survival of each crossbreed family.

Family	Percent Survival ¹ (Laboratory)	Percent Survival ² (Field) – Oct. 1998	Percent Survival (Field) – Dec. 1998	Percent Survival (Field) – Mar. 1999
PG-14	100	82	79	79
PG-2	89	87	87	85
PG-6	85	80	80	80
PG-1	71	66	66	66
PG-18	47	32	32	31
PG-26	42	27	22	22
PG-20	41	23	23	23
PG-21	29	3	3	3
PG-24	26	42	39	38
PG-10	23	33	30	30

¹Laboratory percent survival is reflective of cumulative survival from June through August 1998.

²Field percent survival is reflective of survival from June 1998 through March 1999.

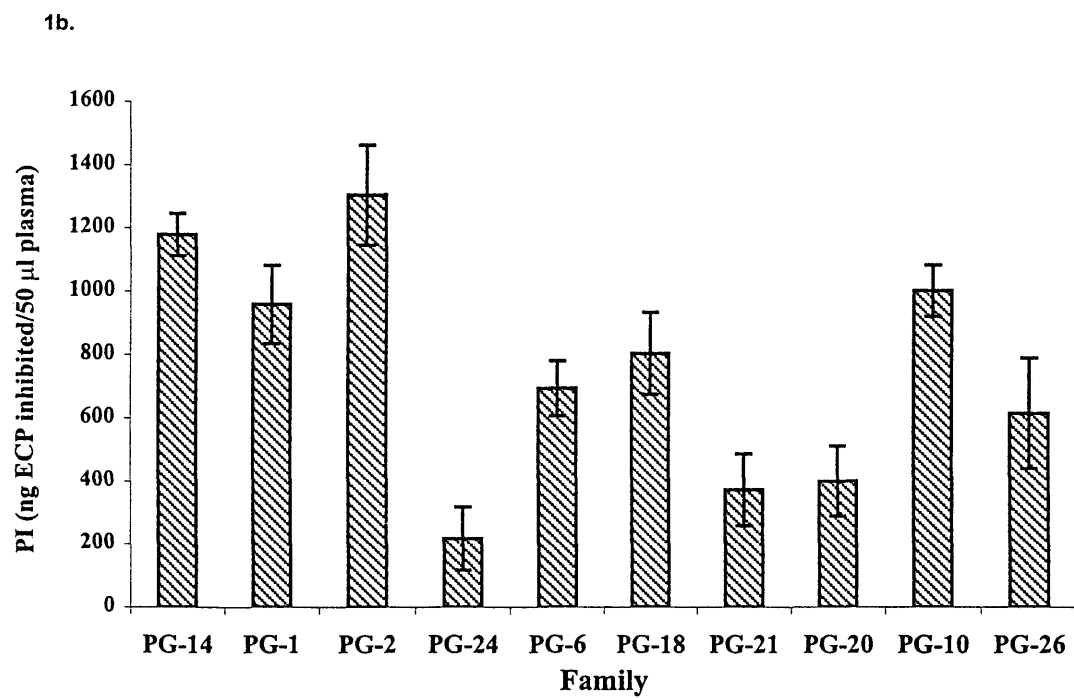
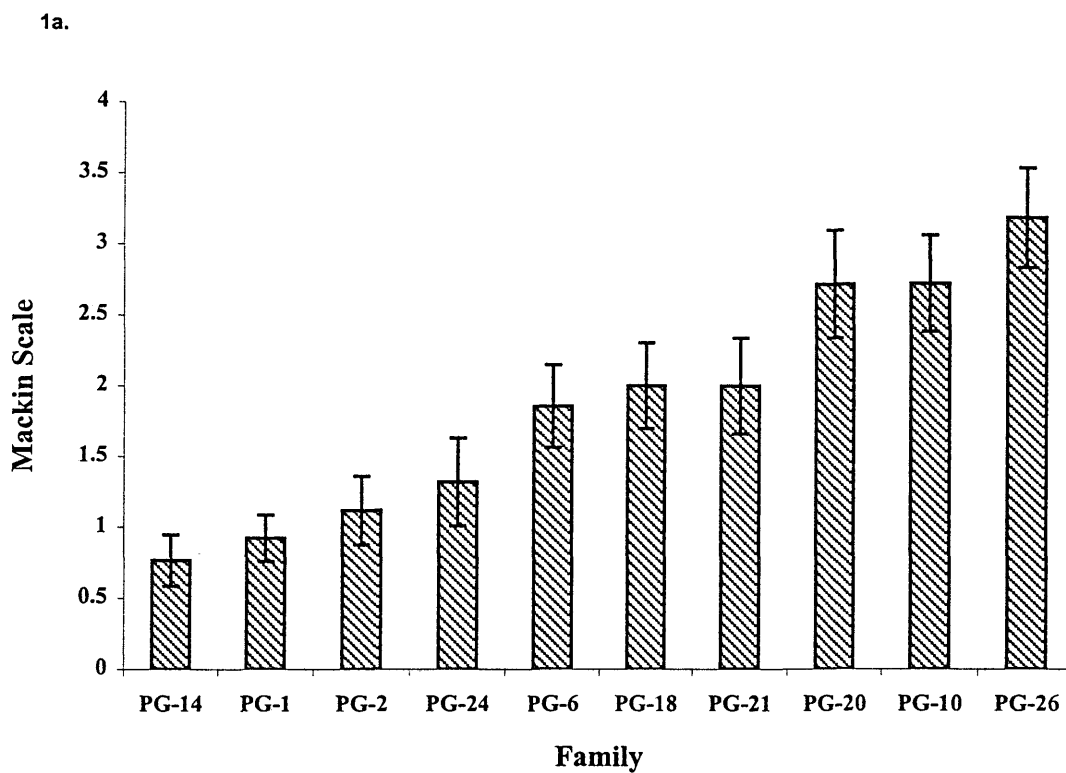
Figure Legends

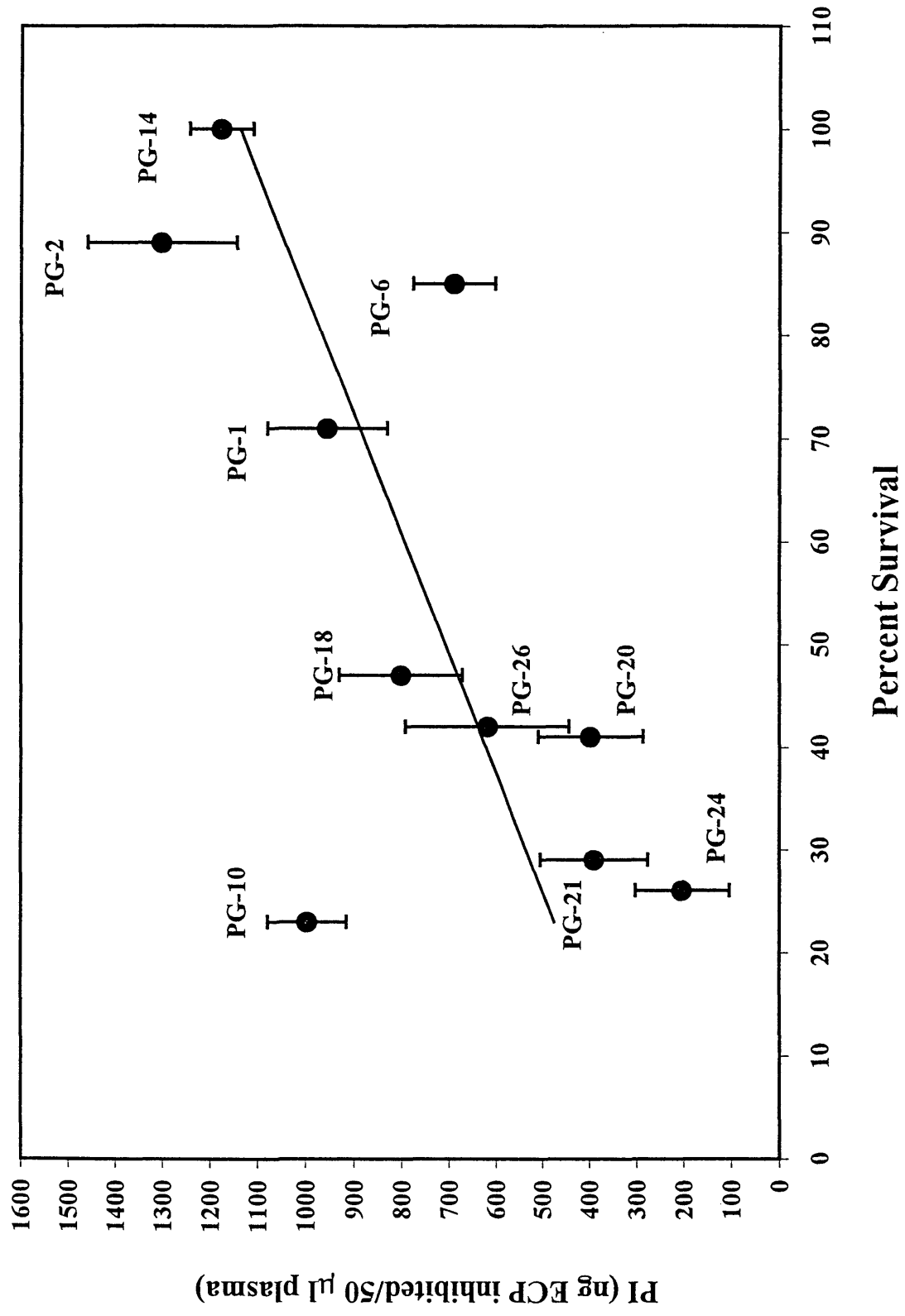
Figure 1a. Levels of hyphospore number per family. Bars represent mean disease intensity for laboratory held oysters. Error bars represent standard error. Values for the Mackin Scale are: 0 – negative, 0.5 – rare, 1.0 – light, 2.0 – light to moderate, 3.0 – moderate, 4.0 – moderate to heavy, 5.0 – heavy. (Mackin, 1962).

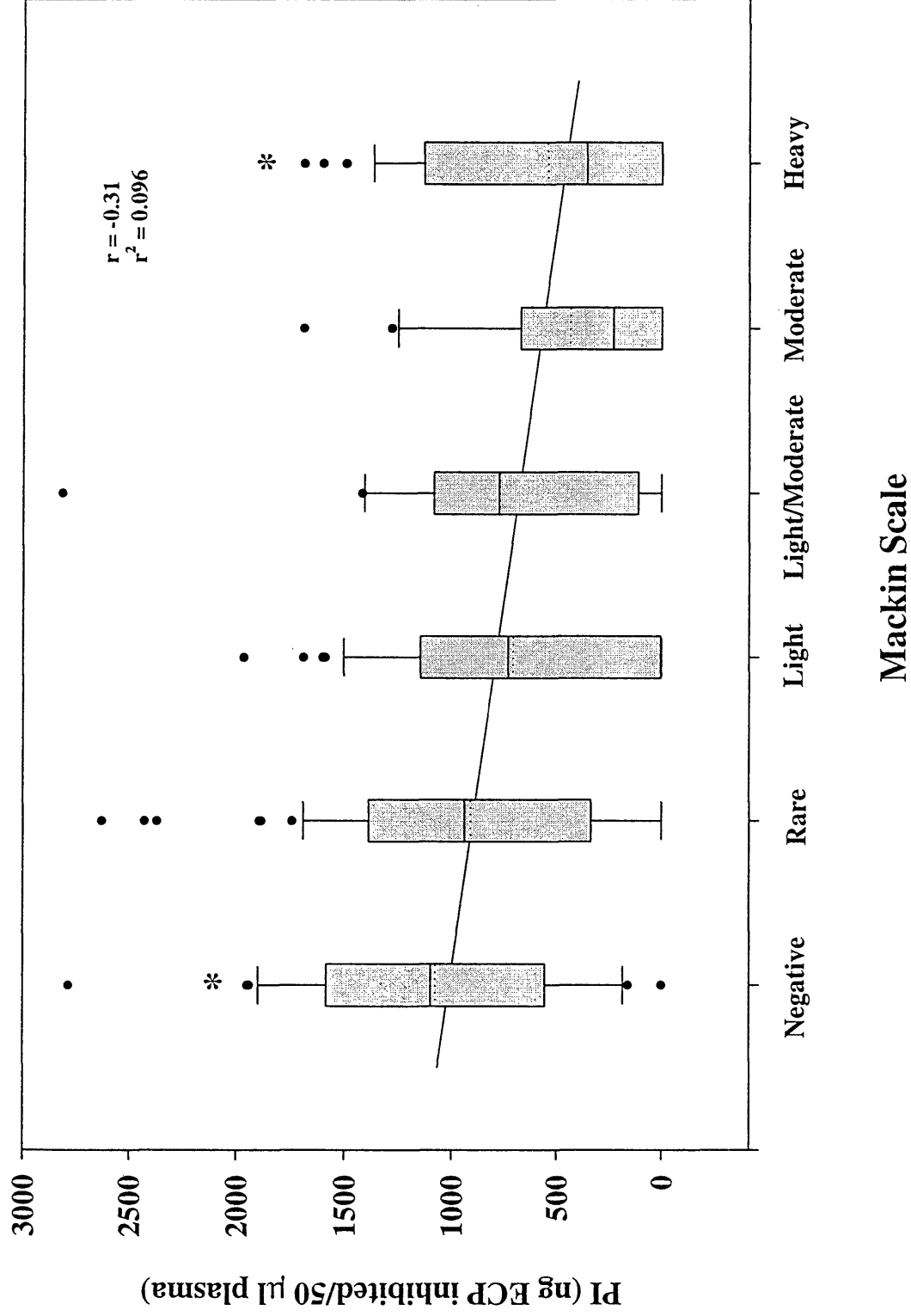
Figure 1b. Levels of low molecular weight (LMW) PI. Bars represent mean PI (ng ECP inhibited/50 μ l plasma) for laboratory held oysters. Error bars represent standard error.

Figure 2. Relationship between mean family PI value and family survival (June through August 1998). Error bars = standard errors of mean PI values. The correlation between family mean PI and survival was significant ($r = 0.685$, $p = 0.029$)

Figure 3. Correlation between Mackin Scale and PI for June and August 1998. Individual oyster PIs were plotted versus their respective Mackin Scale scoring irrespective of family. Shaded boxplots represent the 25th and 75th percentile. The solid line within each boxplot represents the median and the dotted line represents the mean. Plotted points represent data that lie outside the 5th and 95th percentile (error bars). Results indicated a significant negative correlation ($r = -0.31$, $p < 0.0005$), however, the correlation coefficient was low ($r^2 = 0.096$). Asterisks denote a significant difference in PI between negatively and heavily infected oysters ($p < 0.005$ and $p = 0.005$, respectively) via one-way ANOVA and Tukey's multiple comparisons test.







Chapter 3

Protease inhibitory activity to the oyster pathogen, *Perkinsus marinus*,
in selected stocks of eastern oysters, *Crassostrea virginica*

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Abstract

A selectively bred eastern oyster stock (*Crassostrea virginica*), exhibiting marked resistance to the two primary oyster pathogens of the Chesapeake Bay, *Haplosporidium nelsoni* (MSX) and *Perkinsus marinus* (Dermo), as well as unselected stocks were analyzed for protease inhibitory (PI) activity to virulence factors of *P. marinus* to determine if any correlations could be discovered between disease susceptibility to Dermo and PI activity. Disease susceptibility was quantified by the degree of survival and the level of *P. marinus* intensity. These parameters and PI activity were measured in an F4 generation of MSX/Dermo-resistant stock originating in Delaware Bay (DEBY), and two, F1 generation endemic control stocks originating in Mobjack Bay, VA (MOBY) and Tangier Sound, VA (TAGR). Samplings were conducted at three salinity sites in the spring and summer to determine if these environmental conditions would result in differences in *P. marinus* infection intensity. Results showed that the resistant stock (DEBY) exhibited higher growth and survival, and maintained lighter *P. marinus* infections than the control stocks at all sites and times. PI activity, however, was variable among all groups, and DEBY did not consistently exhibit higher PI activity with respect to the control stocks. As disease intensity increased over time, PI activity decreased in all stocks with the exception of the stocks held in the high salinity regime (30+ ppt). Furthermore, PI activity was found to negatively correlate with disease intensity, within and among all stocks.

Introduction

Perkinsus marinus is a protozoan pathogen of the eastern oyster, *Crassostrea virginica*, and is the causative agent of Dermo (Andrews, 1988). Over the past 30 years this pathogen, along with *Haplosporidium nelsoni*, the causative agent in MSX (Ford and Haskin, 1982) has had a severe economic impact on the commercial oyster fishery in the Chesapeake Bay. Between 1960 and 1994, commercial landings fell from 3 million bushels per year to less than 100,000 bushels per year (Burreson and Ragone Calvo, 1996). Strategies to restock oyster populations have included producing and planting putatively disease resistant oysters from selective breeding programs. For example, stocks of oysters that survived and grew unhindered under heavy MSX exposure were bred with other high performance stocks in an effort to propagate resistant oysters (Haskin and Ford, 1979). Moderate success has been made in selecting and propagating MSX-resistant oysters in the Chesapeake Bay, however, there has been little success in breeding Dermo-resistant lines through similar programs (Gaffney and Bushek, 1996).

One impediment in producing Dermo-resistant lines has been the inability to simultaneously select for resistance to both pathogens. It has been observed that co-resistance does not always arise through selective breeding (Gaffney and Bushek, 1996). Rather, decreased susceptibility that arises to one pathogen can be accompanied with continued susceptibility to the other pathogen. Examples of the latter have been observed in MSX-resistant stocks that continue to express susceptibility to Dermo (Chintala and

Fisher, 1989; Bureson, 1991). These results, however, were not unprecedented since selection for resistance to one disease does not necessitate resistance to another disease (Fevolden et al., 1992). Conversely, Ragone-Calvo et al. (1997) demonstrated decreased susceptibility of selectively bred oysters to both *H. nelsoni* and *P. marinus*. A Delaware Bay (DEBY) F3 generation was found to have significantly lower mortality and faster growth than control oysters susceptible to both pathogens.

Another impediment to the production and verification of Dermo-resistant lines is the lack of knowledge of the factors that constitute Dermo resistance in oysters. Underlying factors of disease resistance may be genetic, but resolution of that would require access to a phenotypically diverse population with respect to such a trait. It is thought that by attempting to generate a broad repertoire of phenotypes in oysters, the chances of distinguishing resistant stocks would be greater (Gaffney and Bushek, 1996). However, a selection parameter such as survival has limitations due to the slow growth of oysters and the chronic nature of Dermo. Therefore, markers for disease resistance that can be identified early in the development of an oyster would be attractive alternatives.

Protease inhibitors have received recent attention as important defensive factors against invading pathogens of finfish (Freedman, 1991; Ellis, 1991; Sleasman et al. 1999) and have been implicated as important humoral defense factors in a number of marine molluscs (Armstrong and Quigley, 1992; Bender and Bayne, 1992; Thorgersen et al., 1992; Elsayed et al. 1999). One defensive role of protease inhibitors is neutralization of pathogen proteases. This is particularly germane to Dermo in eastern oysters as serine proteases of *P. marinus* have been detected *in vitro* (La Peyre and Faisal, 1995; La Peyre et al., 1995) and *in vivo* (Ottinger et al., unpubl. data) and Garreis et al. (1996) have

shown that proteases of *P. marinus* could impair certain oyster defense parameters *in vitro*. Thus, their role in oyster humoral defense has been suggested by Faisal et al. (1998) and Oliver et al. (1999). Additionally, protease inhibitory activity (PI) has been correlated with disease intensity in selectively bred oyster families (Oliver, M.S. thesis, chapter 2).

The production of Dermo-resistant oysters would provide an opportunity to study the relationship between PI activity and disease susceptibility. Efforts by Ragone-Calvo et al. (1997) have led to the production of another MSX/Dermo-resistant DEBY F4 generation (pers. comm., Ragone-Calvo, 1999). This stock and two control stocks were deployed for field trials from 1997-1999 at three different salinity regimes (low, moderate, and high). Preliminary data showed the resistant stock, DEBY, to have significantly greater growth and survival than the control stocks (Ragone-Calvo, Calvo, and Burrenson, pers. comm.) at all salinity sites. Additionally, intra-specific Dermo-disease intensity of DEBY was significantly lower than control stocks. Other than growth, survival, and disease intensity, the development of other correlates of resistance have not as yet been attempted.

This study was initiated to determine if a relationship between PI activity and disease susceptibility existed in the three stocks of oysters described above.

Materials and Methods

Oysters

From August 1997 to July 1999, an F4 generation of oysters (DEBY), originating from the selective breeding of an MSX/Dermo-resistant line at VIMS (DB) (Ragone-

Calvo et al., 1997) were evaluated along with two indigenous F1 generations of oysters from Mobjack Bay (MOBY) and Tangier Sound (TAGR), Virginia. Oysters were deployed in three separate salinity regimes in the Chesapeake Bay (high salinity, 30+ ppt – Burton Bay, VA, near Wachapreague, VA; moderate salinity, 15-25 ppt – York River, VA, Gloucester Point; low salinity, 10-15 ppt – Great Wicomico River, VA) and monitored for percent survival, shell height, and prevalence of *P. marinus*. This study was performed in conjunction with the monitoring of the three stocks between May and July 1999. Growth (shell height in mm) and mortality were measured in May and July at all sites. Twenty oysters from each stock were sampled from each salinity site in May and July 1999 to assess environmentally-dependent differences in Dermo intensity. Oysters were notched and 1 ml of hemolymph drawn for PI determination and oysters sacrificed to determine the prevalence and intensity of *P. marinus* infections.

Hemolymph

Oysters were notched using a radial saw and bled for hemolymph. One milliliter aliquots of hemolymph were removed from oyster adductor muscles using 5-cc syringes and 25-gauge needles. Hemolymph samples were centrifuged at 4 °C at 400 x g to pellet the cells and debris. Cell-free hemolymph (plasma) was filtered through a 0.22- μ m syringe-top filter (Costar Scientific Corp., Cambridge, MA). Plasma was centrifuged in a Microcon-10 apparatus (Amicon Inc., Beverly, MA) to isolate low molecular weight plasma components (<10 kDa). The filtrate was frozen at -20°C until further use.

Dermo Diagnosis

Immediately following bleeding, oysters were shucked and gill, mantle, and rectal tissues were sampled for presence of *Perkinsus marinus*. Diagnosis was performed using Ray's Fluid Thioglycollate Method (RFTM) (Ray, 1952, 1966) and infections scored according to the method described by Mackin (1962).

Protease Inhibitory Activity

Protease inhibitory activity within oysters was detected and quantified using a modified Hide Powder Azure method described by Bender et al. (1992). A standard curve of *P. marinus* proteolytic activity was prepared in a 96 well microtiter plate (Costar) using undiluted, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, and 1:128 dilutions of extracellular proteins (ECP) containing serine protease. ECP was derived from *in vitro* propagated cultures of *P. marinus* following the procedure of La Peyre et al. (1995). All dilutions used in the standard curve were made using cold (4°C) artificial seawater (Forty Fathoms Marine Mix Artificial Sea Water, 22 g/L) and kept on ice. Four microliters of each dilution from the standard curve were added to wells in triplicate followed by 50 µl of room temperature artificial seawater. Fifty-four microliters of room temperature artificial seawater were added as a blank. Four microliters of undiluted protease were added to separate triplicate wells followed by addition of 50 µl low molecular weight oyster plasma. Negative controls consisted of 54 µl plasma only. Mixtures of protease and plasma were incubated at room temperature for 15 minutes on a shaker. Following incubation, 100µl of Hide Powder Azure substrate suspension (0.02 g/ml, 150 mM Tris, 30 mM CaCl₂, 0.05% Brij, 20% sucrose, pH 7.5) was added to each well. Plates were incubated at 37°C on a shaker for an additional 3 hours. After incubation, 100 µl of cold

artificial seawater were added to each well to stop the reaction. Plates were centrifuged at $2700 \times g$ for 15 minutes at 4°C . One hundred microliters of supernatant were removed from each well and transferred to another 96 well microtiter plate. Plates were read at 540 nm on a Titertek[®] Multiskan[®] MCC/340 plate reader (Labsystems, Finland) using DeltaSoft 3 software (BioMetallics, Inc., Princeton, NJ). Based on the protease standard curve, reduction in A_{540} due to the inhibition by LMW *C. virginica* plasma was calculated and expressed as ng ECP inhibited/50 μl LMW plasma.

Statistics

Mean shell height, percent survival, RFTM, and PI activity were calculated and plotted for each stock at all sites. Shell height and percent survival data was also expressed as the change in shell height (mm) and percent increase in mortality respectively. RFTM and PI activity were compared for all stocks via paired t-tests ($p = 0.05$) (Minitab, Minitab, Inc., State College, PA). The PI activity and RFTM for individual oysters, irrespective of stock and site, was plotted and linearly regressed. Percent survival and mean PI activity for each stock was plotted and compared using a linear regression (Sigma Plot 4.0, SPSS, Inc., Chicago, IL). Mean stock-specific PI and RFTM were plotted and linearly regressed.

Protein Determination

Protein determinations were made for July plasma samples using the BCA Protein Assay Kit (Pierce, Rockford, IL). Mean protein concentrations (mg/ml) were plotted for

each stock within each site. Additionally, individual oysters were pooled according to their RFTM value and plotted against their respective protein concentration.

Results

Growth and Survival

Growth (shell height in mm) of each stock is shown in Figures 1a and 1b. The MSX/Dermo-resistant line, DEBY, exhibited the best growth at all sites from May to July with the exception of the YR TAGR stock that exhibited slightly better growth than YR DEBY (Figure 1b). The greatest increase in growth for DEBY occurred at the GWR site.

In terms of mortality, DEBY exhibited lower mortality than the control stocks at all sites from Nov. 1998 to May 1999 and May to July, 1999 (Figure 2a). The percent mortality of DEBY from May to July was also lower than the control stocks at all sites with the exception of YR MOBY where the percent mortality was the same (Figure 2b).

Dermo Disease Intensity

Dermo disease intensity and prevalence was measured for each stock at all sites and plotted as mean intensity on the Mackin Scale (Figure 3a). Results show DEBY to have the lowest intensity in May and July at all sites compared to the control stocks. In May, DEBY did not exhibit an infection intensity above that of a rare infection and the prevalence was greatest at 25% (BB). In July, infections intensified slightly and the prevalence increased in DEBY at all sites, however, infection intensity did not rise above rare or light infections. In a similar manner, the control stocks displayed low infection intensity and prevalence in May. However, in contrast to DEBY, infection intensity

increased dramatically in July. Minimally, infections rose to light/moderate infections (GWR MOBY, BB TAGR) and maximally, rose to moderate infections (GWR TAGR, YR TAGR). Intra-stock paired t-tests were performed and results indicated significant increases in disease intensity ($p < 0.05$) at each site for all stocks. Over the course of the study, one-way ANOVA showed infections in DEBY were significantly lower ($p < 0.001$) than the control stocks at GWR and YR, thus supporting our hypothesis that DEBY would exhibit less infection intensity than the controls. However, at BB the difference between DEBY and the controls was not significant ($p = 0.087$).

Protease Inhibitory Activity

Protease inhibitory activity was measured for each stock at all sites and plotted as mean PI (Figure 3b). Results showed variability among stocks for May and July. DEBY did not show uniformly greater PI than the control stocks either in May or July with one exception. At GWR in July, DEBY exhibited significantly higher PI than the controls. DEBY exhibited greater PI at YR in May; however, it was not significantly different from the controls.

PI activity did not increase over time as disease intensity increased. Rather, PI activity decreased from May to July in all groups with the exception of BB MOBY and TAGR where PI increased. Paired t-tests were performed at each site for each stock to determine differences between May and July time points. The control stocks exhibited significant decreases in PI activity ($p < 0.05$) at GWR and YR. In contrast, PI activity in the DEBY stock did not decrease significantly at either site. At BB, PI activity increased significantly ($p < 0.05$) for MOBY, but the increase was not significant for TAGR. DEBY

PI decreased, however, the result was not significant. There appeared to be a salinity effect on PI in May as BB PI was lower for all groups compared to GWR and YR. The salinity effect appears to have diminished in July as PI was not considerably different from the other sites.

Correlation of PI Activity with Survival and RFTM

Individual oysters were scatter plotted according to their respective PI activity and RFTM and the result linearly regressed (Figure 4). A trend of higher PI with lower RFTM was observed

($r = -0.25$) ($p < 0.0001$), however, the correlation coefficient was too low to suggest any predictable relationship between PI and disease intensity ($r^2 = 0.06$).

Mean stock PI and percent survival were plotted and the result linearly regressed (Figure 5). The low correlation coefficient ($r^2 = 0.02$) indicated that there was no relationship between the two. A slight negative correlation was detected ($r = -0.14$), however, the result was not significant.

Mean stock PI and disease intensity on RFTM were plotted and the result linearly regressed (Figure 6). The correlation coefficient was low ($r^2 = 0.13$) suggesting that PI accounts for only a small proportion of the variability in disease intensity. A negative correlation between PI and disease intensity was observed ($r = -0.36$).

Protein Determination

Oysters from GWR and YR sites exhibited higher protein concentrations than oysters from the BB site (Figure 7). This result is not surprising and it is likely a salinity effect

as each of these stocks were bred for an optimal salinity below that observed at BB (Calvo, pers. Comm.). No significant differences were detected between stocks within each site. When individual oysters protein values were plotted with respect to their RFTM, no significant differences were observed (Figure 8). Unlike PI activity, there was no apparent trend of lower protein concentration with higher Dermo intensity.

Discussion

This study was initiated to determine whether a biochemical factor, such as protease inhibitory activity, could be directly correlated with Dermo disease resistance. The parameters used to measure resistance are based solely on survival and the degree of infection. At present, there are no genetic or biochemical factors or predictors of Dermo resistance. There is, however, strong evidence that the conferring of Dermo resistance is genetic (Gaffney and Bushek, 1996). Most of this evidence comes from the decades of selective breeding practices of oysters resistant to MSX (Haskin and Ford, 1979; Ford and Haskin, 1987; Gaffney and Bushek, 1996).

Understanding the mechanisms of pathogenesis can lead to the identification of important host defense markers. La Peyre and Faisal (1995) and La Peyre et al. (1995) observed the secretion of serine proteases by *P. marinus* into the culture medium and speculated that they may be important molecules in the pathogenesis. Previous work by Oliver et al. (1999) demonstrated that eastern oysters possess low molecular weight inhibitory activity directed against these suspected virulence factors of *P. marinus*. Specifically, low molecular weight plasma constituents (8-10 kDa) were observed to inhibit *P. marinus* serine protease digestion of a specific host plasma protein *in vitro*.

Based on this observation, it was hypothesized that the observed anti-proteolytic activity in eastern oysters might serve as a biochemical marker for Dermo resistance.

The selective breeding of MSX/Dermo-resistant oysters (DEBY) at VIMS provided us with an opportunity to correlate PI with Dermo resistance. In addition, the production of two control stocks endemic to the Chesapeake Bay which were believed to be susceptible to *P. marinus* (MOBY and TAGR) provided an important comparison. It was our intention to determine if a correlation between PI activity and disease susceptibility existed in each stock. Survival and Dermo disease data supported previous studies suggesting that DEBY exhibits apparent disease resistance. DEBY exhibited greater cumulative growth and appeared to be the least susceptible to Dermo of the three strains by exhibiting lower mortality and infection intensity. PI activity, however, did not correlate with these other parameters, as it appeared to be variable. In fact, DEBY PI activity was often lower than that of the controls in May and July. PI activity did not increase over time which might be anticipated if this component were inducible. In fact, PI activity decreased significantly from May to July in the controls at GWR and YR. It is interesting to note that PI activity in DEBY did not significantly decrease at GWR and YR.

The discrepancy between PI activity and survival and infection intensity might lead one to conclude that PI is not a factor in Dermo resistance. However, it is possible that the sensitivity of the assay may have precluded us from accurately determining protease inhibitor activity, and thus may have led to an underestimation of the true activity present in the plasma. First, protease inhibitors of serine proteases operate by binding the active sites of proteases (Laskowski and Kato, 1980). Serine protease inhibitors are often small

molecules (370-390 amino acids in length) (Huber and Carrell, 1989). The PI activity directed against *P. marinus* serine protease activity found in *C. virginica* plasma was also of low molecular weight (8-10 kDa) (Oliver et al., 1999), however, the exact sequence has not been determined. If protease inhibitors of *C. virginica* were binding the active site of *P. marinus* proteases as suggested by the mechanisms described by Laskowski and Kato (1980), this would preclude the assay from detecting already bound protease inhibitors. That is to say, the assay employed herein only detected free or unbound inhibitors. It is possible that as disease intensity increased from May to July, the amount of protease also increased. In response to this, free and unbound inhibitors in the plasma would have become bound to the increasing amounts of *P. marinus* protease and thus, would not be detected using the Hide Powder Azure assay. This would account for the significant decreases in PI activity observed in the control stocks at GWR and YR. The observation that PI activity in DEBY did not significantly decrease from May to July suggests that DEBY was able to maintain higher levels of unbound inhibitor. This may have occurred directly, through induced expression of inhibitors, or indirectly, through a separate suppressive mechanism of *P. marinus* proliferation and protease expression. In either case, the possibility of PI activity being involved in resisting *P. marinus* infections cannot be ruled out. One avenue that can circumvent the possibility of underestimating protease inhibitor levels is the use of a more sensitive assay. This could either be antibody-based where a probe could detect free and bound inhibitor, or molecular-based where mRNA could be detected if such an induction of inhibitor occurred.

These studies are of particular interest in light of previous studies (Oliver, M.S. thesis, chapter 2) wherein a strong statistical correlation existed between the level of PI

activity and resistance. Results of this study suggest a low correlation between PI activity and Dermo intensity and PI and survival.

One reason for the lack of agreement between the results observed by Oliver (1999, M.S. thesis, chapter 2) and the results observed herein with regard to PI activity and Dermo intensity might be related to the genetic variability of the oysters. The former result was observed in ten families of oysters that were produced from oysters with broad geographical origins (see M.S. thesis, chapter 2, Table 1). The resistant oyster stock tested in this study originated from several generations of inbred parents and was compared to control stocks whose parents were simply individuals from endemic oyster stocks in the Chesapeake Bay (Calvo, pers. comm.). The restricted genetic variability in these stocks, compared to the more genetically varied families, may have been insufficient in allowing for a clear relationship between PI activity and disease resistance to be seen. The less significant difference observed herein between PI activity and disease resistance does not preclude the importance of PI activity in oysters. Protease inhibitors, whether as defensive molecules or as targets of *P. marinus* virulence factors, are likely still important but may not be the single critical factor in resistance.

Another reason for the low correlation between PI and survival and Dermo intensity might be the presence of other pathogens in the oyster. For instance, the virulence factors of many oyster parasites such as *H. nelsoni* and *H. coastale* (SSO disease) are not known. It is possible that either through direct or indirect routes, these pathogens compromise PI. This would have no bearing on survival, as the high surviving oysters in this study were already MSX-resistant. Similarly, PI may have been compromised in low *P. marinus* infected oysters. The ability of other pathogens to affect PI might have precluded us

from observing a clear relationship between PI and Dermo intensity. In this way, the use of field challenges to *P. marinus* may not allow for the clear determination of what role PI plays in resistance to Dermo. Alternatively, tightly controlled laboratory infections of uninfected resistant and susceptible oysters using filtered water would resolve the challenge of having confounding pathogens. Nevertheless, a significant negative correlation was apparent between PI and Dermo intensity, both within and among stocks.

Plasma protein concentrations of July oysters was determined and analyzed concurrently with PI activity. This was done to determine if plasma protein concentration could be correlated with Dermo intensity and if there was an effect of plasma protein concentration on PI activity. In contrast to the finding that there was a significant negative correlation between PI activity and Dermo intensity, there was no significant decrease of plasma protein concentration with higher Dermo intensities. Furthermore, there were no significant differences in plasma protein concentration between stocks within each site. However, clear differences in stock-specific RFTM were observed. When PI activity and protein concentrations are considered together, it appears that changes in PI activity do not reflect any significant changes in plasma protein concentration as infection intensity increases. Conversely, this suggests that oysters are capable of maintaining plasma protein concentration at higher Dermo intensities, however, they may not be able to maintain PI activity. Thus, it appears that plasma protein concentration and PI activity are not coupled phenomena during Dermo infections.

The concept of a biochemical factor(s) contributing to increased disease resistance is well established. Ford (1986) examined MSX- resistant and susceptible electrophoretic

hemolymph protein profiles in an attempt to identify protein differences. Additionally, Chintala and Fisher (1989) noted an association of lower susceptibility with higher serum lectin concentrations in MSX-resistant oysters compared to native oysters, although no precise causative relationship was posed. Specifically, protease inhibitors have been identified in a number of molluscan species (Armstrong and Quigley, 1992) and have been implicated in disease resistance in the snail, *Biomphalaria glabrata*, by Fryer et al. (1991). Fryer et al. showed that resistant strains of the *B. glabrata* exhibited significantly higher PI to the trematode, *Schistosoma mansoni*, than did susceptible strains. The endogenous PI may not necessarily be responsible for resistance in strains of *B. glabrata* since the PI was assessed by its activity against commercial proteases and not proteases of *S. mansoni*. Teleost protease inhibitors have also been suggested as important mediators of disease resistance. Ellis (1991) purified an α_2 -macroglobulin-like molecule from rainbow trout (*Oncorhynchus mykiss*) sera and demonstrated its neutralizing activity against a major virulence factor of the pathogen, a protease of *Aeromonas salmonicida*. In a study by Freedman (1991), the α_2 -macroglobulin-like neutralizing activity to *A. salmonicida* in rainbow trout was compared to that of brook trout (*Salvelinus fontinalis*). Freedman found that brook trout exhibited lower activity and is more susceptible to furunculosis.

This use of protease inhibitory activity as a correlate of Dermo resistance is continuing to be explored. The advent of more sensitive assay tools, along with more tightly controlled infection experiments with *P. marinus*, may facilitate our understanding of the role of PI in resisting *P. marinus* infections. Furthermore, they may provide insight into understanding oyster humoral defense and *P. marinus* pathogenesis.

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References

- Andrews, J. D. 1988. Epizootiology of the disease caused by the oyster pathogen *Perkinsus marinus* and its effect on the oyster industry. *Amer. Fish. Soc. Spec. Publ.* **18**:47-63.
- Armstrong, P. B. and Quigley, J. P. 1992. Humoral immunity: α_2 -macroglobulin activity in the plasma of mollusks. *Veliger*. **35**:161-164.
- Bender, R. C., Fryer, S. E., and Bayne, C. J. 1992. Proteinase inhibitory activity in the plasma of a mollusc: Evidence for the presence of α -macroglobulin in *Biomphalaria glabrata*. *Comp. Biochem. Physiol.* **102B**(4):821-824.
- Bender, R. C., and Bayne C. J. 1996. Purification and characterization of a tetrameric α_2 -macroglobulin proteinase inhibitor from the gastropod mollusc *Biomphalaria glabrata*. *Biochem. J.* **315**:893-900.
- Burreson, E. M. 1991. Effects of *Perkinsus marinus* infection in the eastern oysters *Crassostrea virginica* (Gmelin, 1791): I. Susceptibility of native and MSX-resistant stocks. *J. Shellfish Res.* **10**:417-423.
- Burreson, E. M. and Ragone Calvo, L. M. 1996. Epizootiology of *Perkinsus marinus* disease of oysters in Chesapeake Bay, with emphasis on data since 1985. *J. Shellfish Res.* **15**:17-34.
- Chintala, M. M. and Fisher, W. S. 1989. Disease incidence and potential mechanisms of defense for MSX-resistant and susceptible eastern oysters held in Chesapeake Bay. *J. Shellfish Res.* **10**:439-443.
- Ellis, A. E. 1991. Inhibition of the *Aeromonas salmonicida* extracellular protease by α_2 -macroglobulin in the serum of rainbow trout. *J. Fish Dis.* **14**:265-277.

- Elsayed, E. E., McLaughlin, S. M. and Faisal, M. 1999. Effects of neoplasia on plasma protease inhibitory activities in the softshell clam, *Mya arenaria*. *Comp. Biochem. Physiol.* In press.
- Faisal, M., MacIntyre, E. A., Adham, K. G., Tall, B. D., Kothary, M. H., and La Peyre, J. F. 1998. Evidence for the presence of protease inhibitors in eastern (*Crassostrea virginica*) and Pacific (*Crassostrea gigas*) oysters. *Comp. Biochem. Physiol.* **121B**:161-168.
- Fevolden, S. E., Fefstie, T., and Roed, K. H. 1992. Disease resistance in rainbow trout (*Onchorynchus mykiss*) selected for stress response. *Aquaculture.* **104**:19-29.
- Ford, S. E. 1986. Comparison of hemolymph proteins between resistant and susceptible oysters, *Crassostrea virginica*, exposed to the parasite, *Haplosporidium nelsoni* (MSX). *J. Invertebr. Pathol.* **47**:283-294.
- Ford, S. E. and Haskin, H. H. 1982. History and epizootiology of *Haplosporidium nelsoni* (MSX), an oyster pathogen in Delaware Bay, 1957 – 1980.). *J. Invertebr. Pathol.* **40**:118-141.
- Ford, S. E. and Haskin, H. H. 1987. Infection and mortality patterns in strains of oysters *Crassostrea virginica* selected for resistance to the parasite *Haplosporidium nelsoni* (MSX). *J. Parasitol.* **73**:368-376.
- Freedman, S. J. 1991. The role of α_2 -macroglobulin in furunculosis: a comparison of rainbow trout and brook trout. *Comp. Biochem. Physiol.* **98B**:549-553.
- Fryer, S. E., Bender, R. C. and Bayne, C. J. 1991. Proteinase inhibitory activity in the plasma of the gastropod mollusc, *Biomphalaria glabrata*. *Dev. Comp. Immunol.* **15**:S45.

- Gaffney, P. M. and Bushek, D. 1996. Genetic aspects of disease resistance in oysters. *J. Shellfish Res.* **15**(1):135-140.
- Garreis, K. A, La Peyre, J. F, and Faisal, M. 1996. The effects of *Perkinsus marinus* extracellular products and purified proteases on oyster defence parameters *in vitro*. *Fish Shellfish Immunol.* **6**:581-597.
- Haskin, H. H. and Ford, S. E. 1979. Characteristics of inbred oyster strains selected for resistance to *Haplosporidium nelsoni* (MSX). *Mar. Fish. Rev.* **41**:54-63.
- Huber, R., and Carrell, R.W. (1989). Implications of the three-dimensional structure of alpha-1-antitrypsin for structure and function of serpins. *Biochemistry.* **28**, 8951-8966.
- La Peyre, J. F. and Faisal, M. 1995. *Perkinsus marinus* produces extracellular proteolytic factor(s). *Bull. Eur. Assoc. Fish Pathol.* **15**:1-4.
- La Peyre, J. F., Schafhauser, D. Y., Rizkalla, E. H., and Faisal, M. 1995. Production of serine proteases by the oyster pathogen *Perkinsus marinus* (Apicomplexa) *in vitro*. *J. Euk. Microbiol.* **42**:544-551.
- Laskowski, M. and Kato, I. 1980. Protein inhibitors of proteinases. *Ann. Rev. Biochem.* **49**:593-626.
- Mackin, J. G. 1966. Oyster disease caused by *Dermocystidium marinum* and other microorganisms in Louisiana. *Publ. Inst. Marine Sci. Univ. Texas.* **7**:132-229.
- Oliver, J. L., Lewis, T. D., Faisal, M., and Kaattari, S. L. 1999. Analysis of the effects of *Perkinsus marinus* proteases on plasma proteins of the eastern (*Crassostrea virginica*) and Pacific oyster (*Crassostrea gigas*). *J. Invertebr. Path.* **74**:173-183.

- Ragone-Calvo, L. M., Harmon, V., and Burrenson, E. M. 1997. Selection of oysters for resistance to two protozoan parasites. *J. Shellfish Res.* **16**(1):327-328.
- Ray, S. M. 1952. A culture technique for the diagnosis of infection with *Dermocystidium marinum* (Mackin, Owen, and Collier) in oysters. *Science*. **116**:360-361.
- Ray, S.M. 1966. A review of the culture method for detecting *Dermocystidium marinum*, with suggested modifications and precautions. *Proc. Natl. Shellfish Assoc.* **54**:55-69.
- Sleasman, J. W., Nelson, R. P., Goodenow, M. M., Wilfret, D., Hutson, A., Baseler, M., Zuckerman, J., Pizzo, P. A., Mueller, B.U. 1999. Immunoreconstitution after ritonavir therapy in children with human immunodeficiency virus infection involves multiple lymphocytes lineages. *J. Pediatr.* **134**(5):597-606.
- Thøgersen, I. B., Salvesen, G., Brucato, F. H., Pizzo, S. V. and Enghild J. J. 1992. Purification and characterization of an α_2 -macroglobulin from the mollusc *Octopus vulgaris*. *Biochem. J.* **285**:521-527.

Figure Legends

Figure 1a. Mean shell heights (mm) ($n = 100$) for each stock in May (white) and July (black) 1999 at each site (GWR – Great Wicomico River, YR – York River, BB – Burton Bay). Error bars represent the standard error of the mean.

Figure 1b. Mean increase in shell height (mm) from May to July 1999. Increase was based on mean shell heights from May and July ($n = 100$).

Figure 2a. Percent survival for each stock from Nov. 1998 to May 1999 (white) and from May to July 1999 (black).

Figure 2b. Percent mortality for each stock from May to July 1999.

Figure 3a. Mean Dermo disease intensity for each stock as rated on the Mackin Scale. Disease intensity was measured for each stock from a subsample of 20 oysters per stock in May (white) and July (black) 1999. Error bars represent the standard error of the mean. Percentages above each bar reflect the percent prevalence of *P. marinus* within each stock. Ratings on the Mackin Scale are noted on the figure. Results showed significant differences for every stock at each site ($p < 0.05$).

Figure 3b. Mean plasma protease inhibitory activity (PI) (ng ECP inhibited 50 μ l plasma) for each stock in May (white) and July (black) 1999. Error bars represent the standard error of the mean. Asterisks denote significant differences ($p < 0.05$). One-way

ANOVA was performed to compare PI within site. Significant differences are denoted by # ($p < 0.05$).

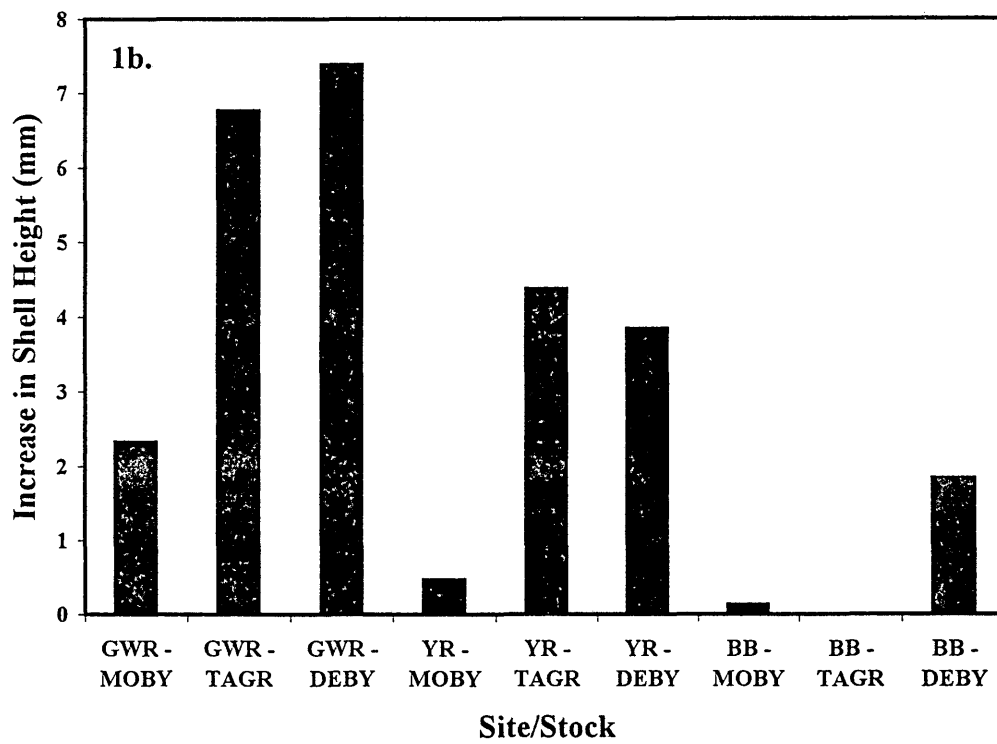
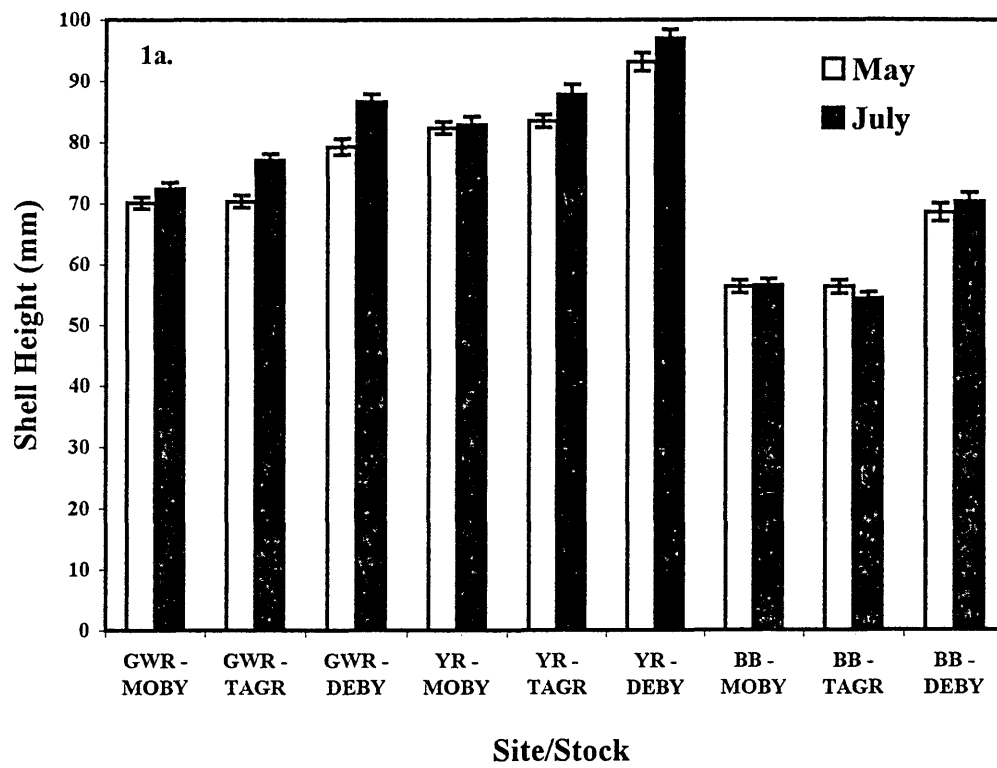
Figure 4. Scatter plot of individual oyster mean PI (ng ECP inhibited 50 μ l plasma) and Dermo intensity as rated on the Mackin Scale. Oysters were plotted irrespective of stock. The shaded box represents the 25th and 75th percentiles. The solid line within the box represents the median the dotted line represents the mean. Error bars represent the 10th and 90th percentiles. Linear regression of PI and RFTM resulted in a negative correlation ($r = -0.25$) and a low correlation coefficient of 0.06. The result, however, was significant ($p < 0.0001$).

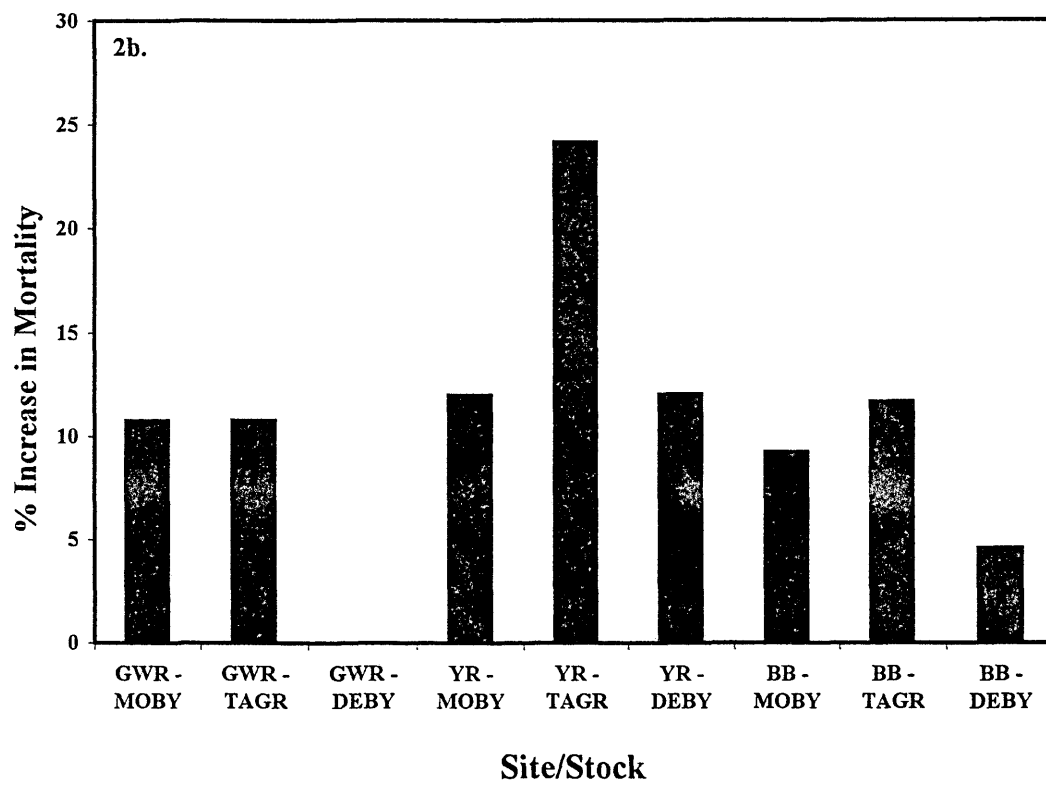
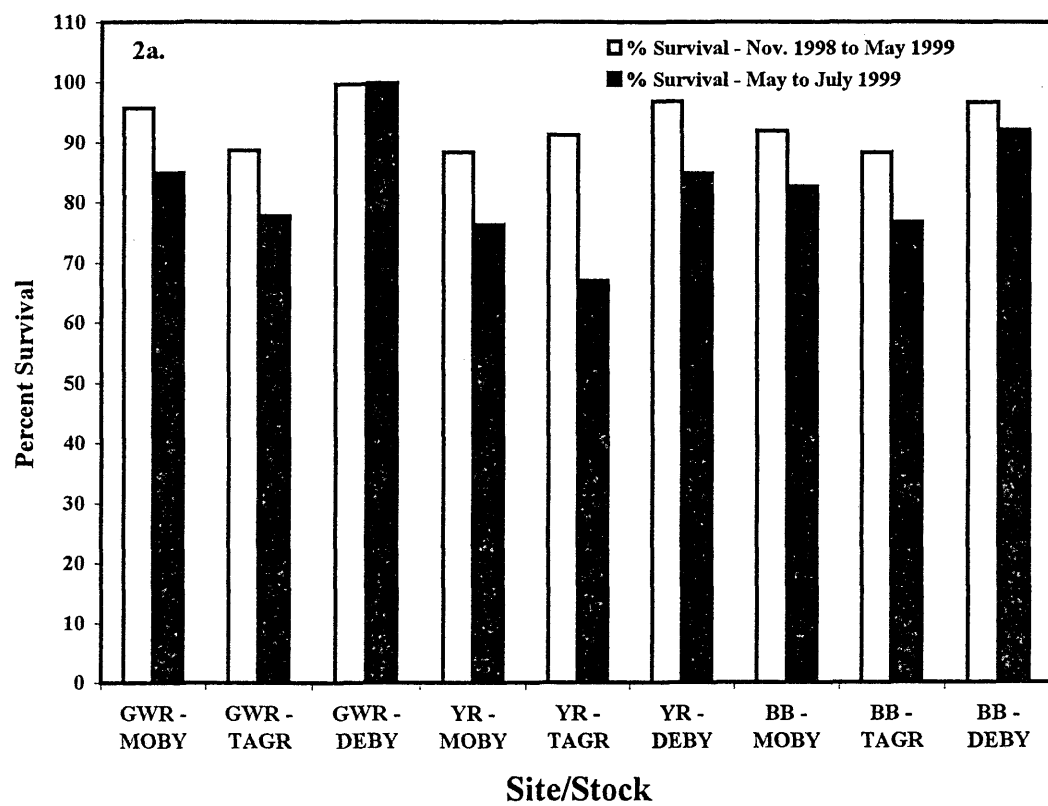
Figure 5. Mean stock PI (ng ECP inhibited 50 μ l plasma) versus stock survival. Error bars represent the standard error of the mean. Linear regression of mean PI and survival resulted in a slight negative correlation between the two ($r = -0.14$), however the correlation coefficient was low ($r^2 = 0.02$). The result was not significant at $p = 0.05$.

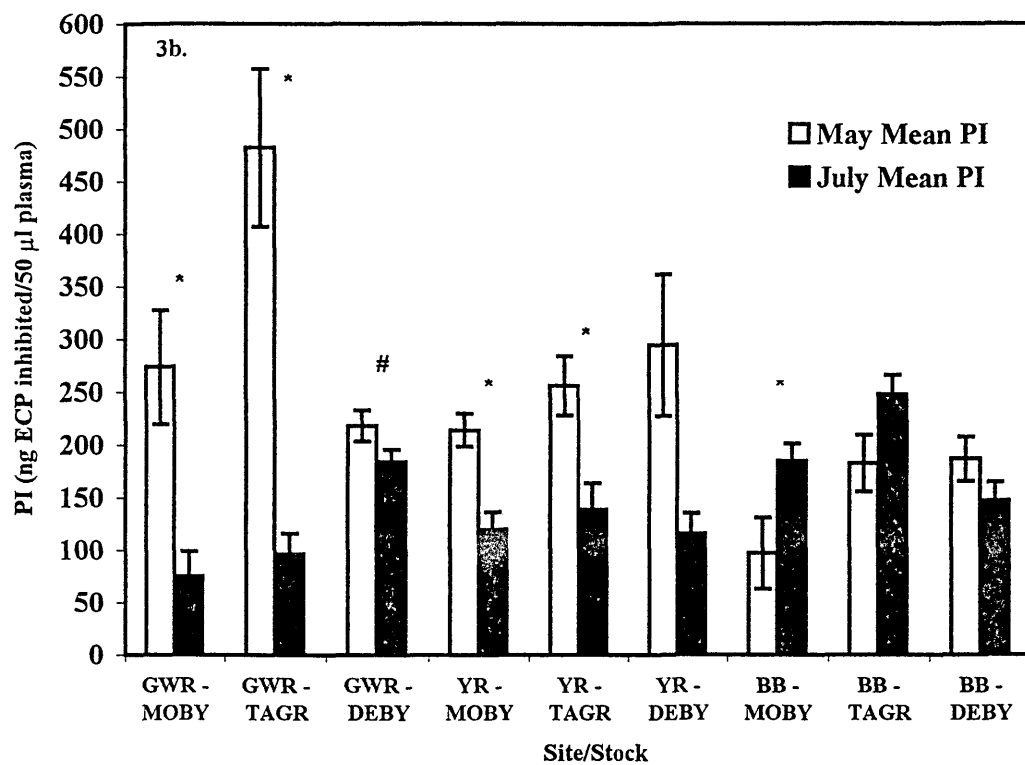
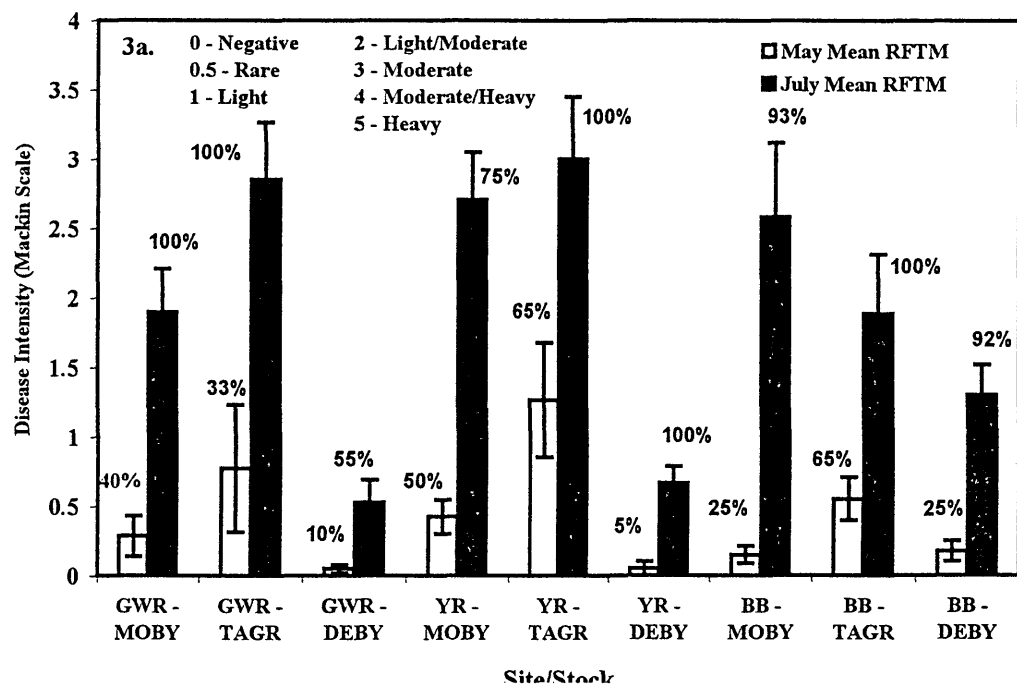
Figure 6. Mean stock PI (ng ECP inhibited 50 μ l plasma) versus mean stock RFTM. Error bars represent the standard error of the mean. Linear regression of PI and RFTM resulted in a negative correlation between the two ($r = -0.36$) with a correlation coefficient of 0.13. The result was not significant at $p = 0.05$. Ratings on the Mackin Scale are noted on the figure. Asterisks denote the MSX- and Dermo-resistant stock, DEBY.

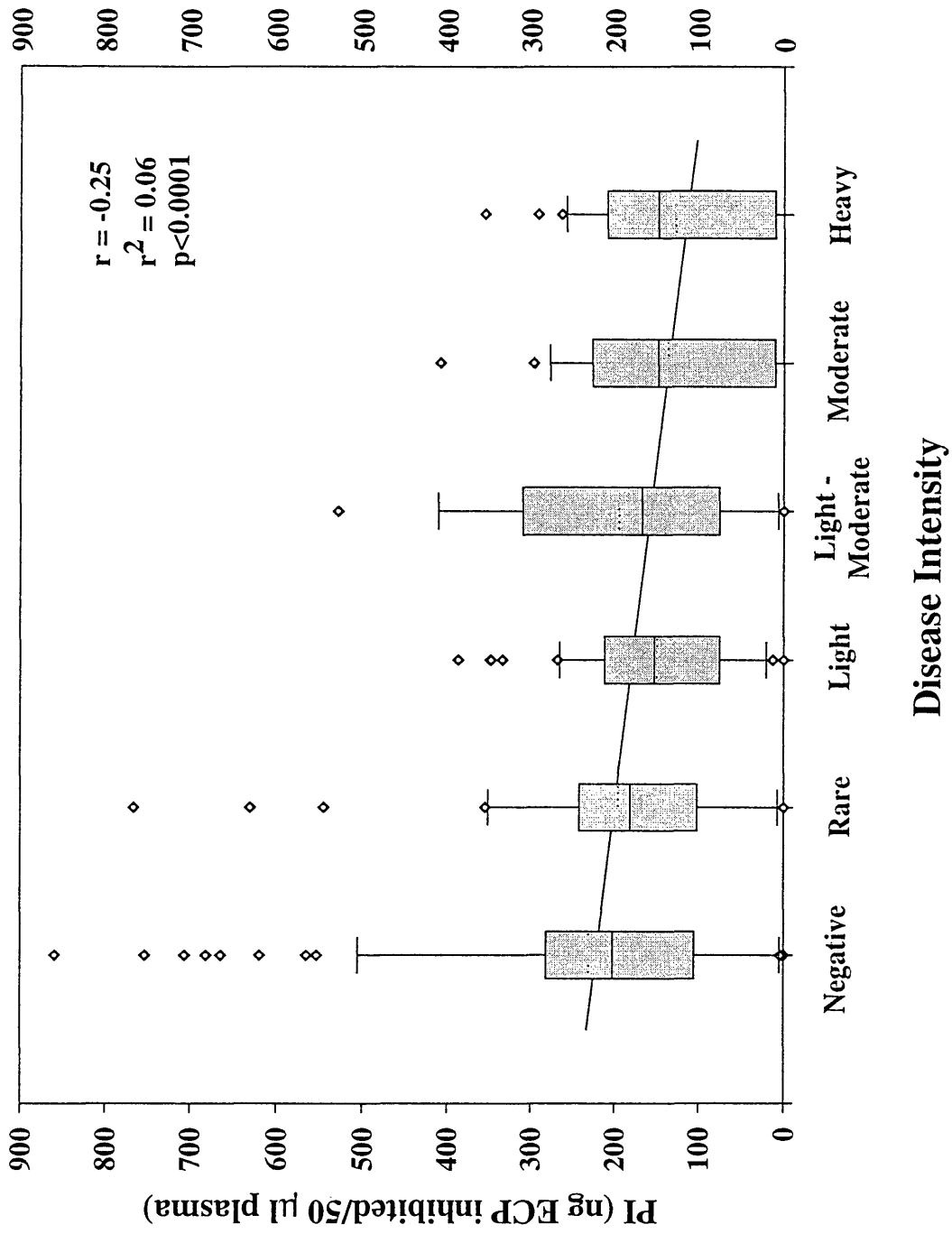
Figure 7. Mean plasma protein concentrations for each stock in July 1999. Error bars represent the standard error of the mean.

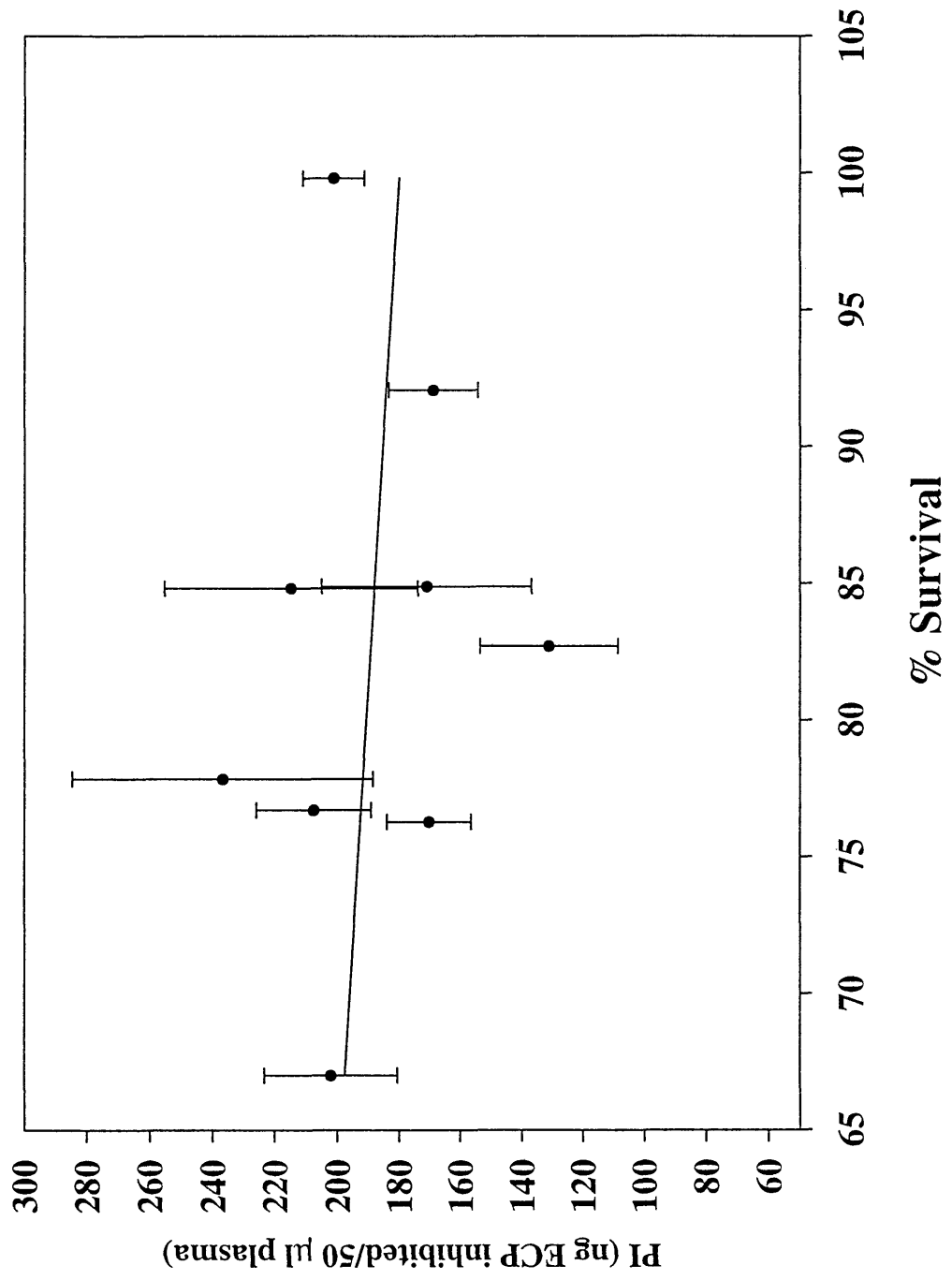
Figure 8. Mean plasma protein concentration for each RFTM rating for July 1999 oysters. Individual oyster protein concentrations were grouped according to its RFTM rating and means calculated for each rating. Error bars represent the standard error of the mean. No significant differences were detected.

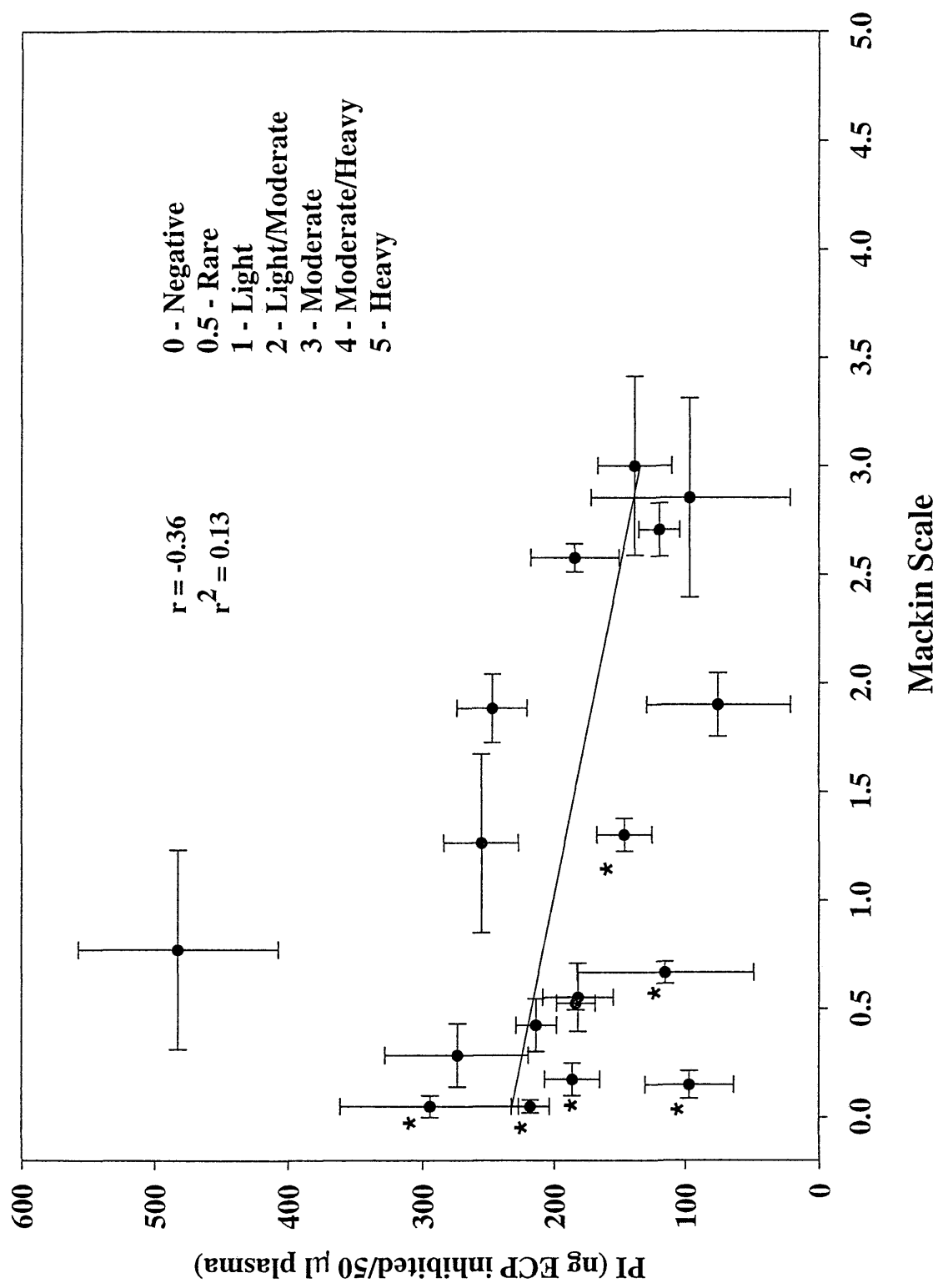


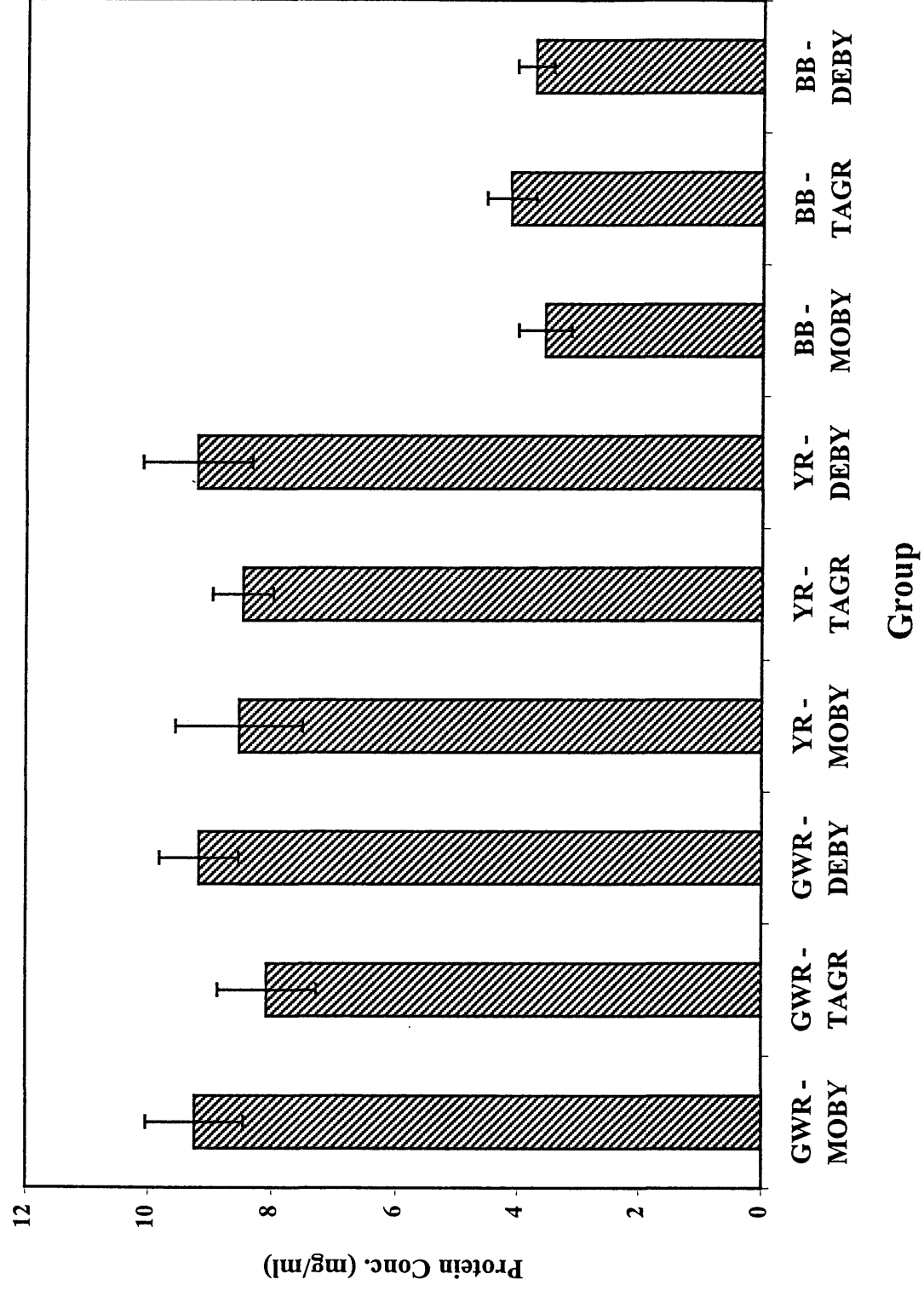


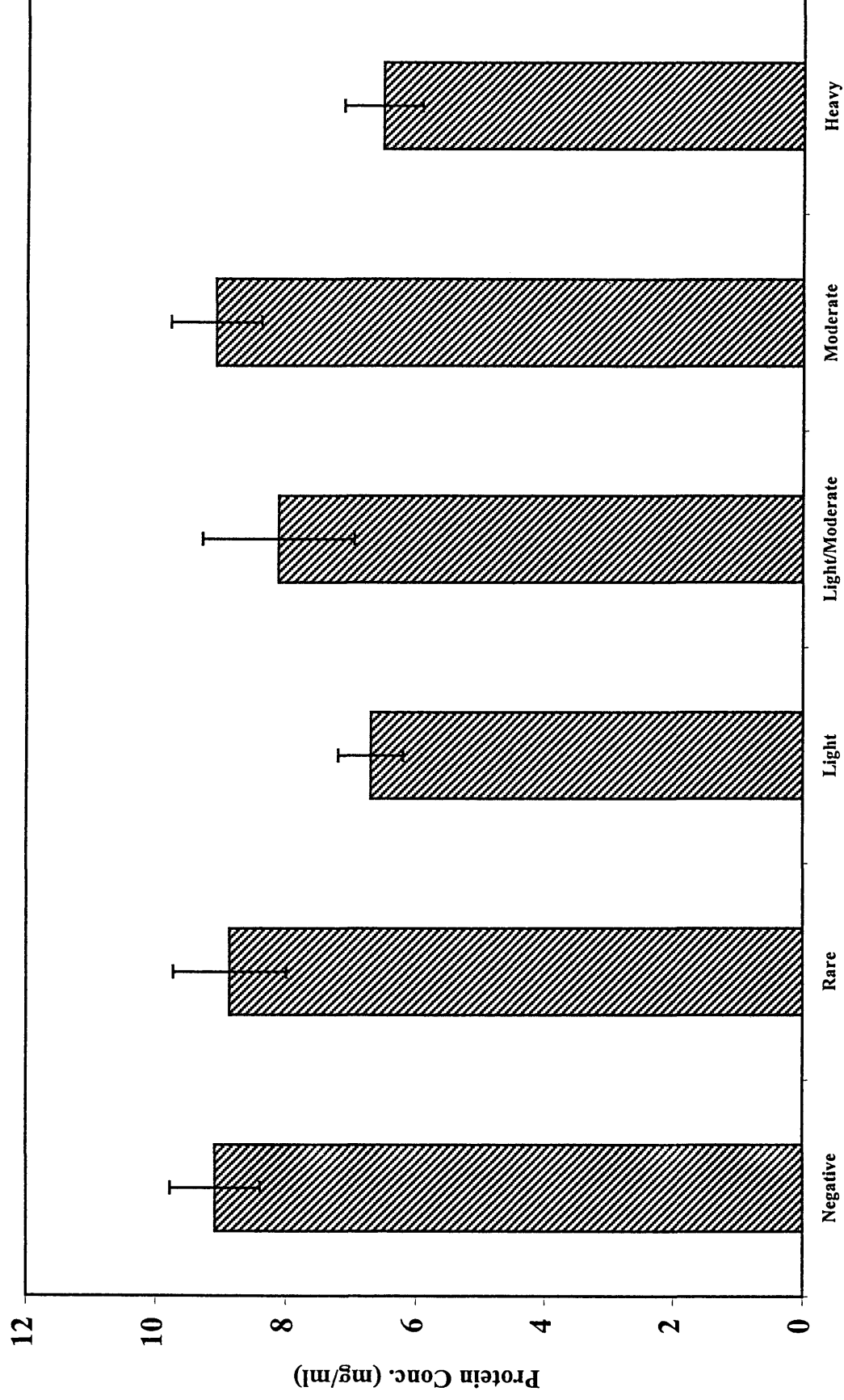












Disease Intensity (Mackin Scale)

Chapter 4

In vivo induction of protease inhibitory activity
in eastern oysters, *Crassostrea virginica*,
infected with *Perkinsus marinus*

Abstract

Three groups of eastern oysters, *Crassostrea virginica*, were monitored for protease inhibitory activity (PI) after challenge with the oyster pathogen, *Perkinsus marinus*. One group was challenged with *in vitro* cultured *P. marinus* (1×10^6 viable meronts/ml), the second group was challenged with formalin-treated/heat-inactivated *P. marinus* (1×10^6 meronts/ml), and the third group was challenged with sterile-filtered artificial seawater. Hemolymph was obtained at 3 weeks prior to challenge and weeks 1, 3, and 5 post-challenge (p.c.) and measured for low molecular weight PI. PI was compared to baseline levels (3 weeks pre-challenge) of PI for each treatment. Disease intensity for each treatment was measured via Ray's Fluid Thioglycollate Method (RFTM) following the final bleed at week 5. The treatment receiving live *P. marinus* exhibited, on average, rare to light infections. The other two treatments were negative for *P. marinus* infections. PI activity was analyzed in two ways: 1. PI activity was measured and standardized to plasma protein concentration (specific PI activity), 2. PI activity was measured with respect to the volume of plasma assayed (non-specific PI activity). Results of PI analysis indicate that PI is inducible upon challenge with live and killed *P. marinus* as evidenced by a significant increase in specific PI activity at week 1 p.c. Furthermore, at weeks 3 and 5 p.c. specific PI activity remained significantly higher than the baseline in the treatment receiving killed *P. marinus*, whereas PI activity dropped to near baseline levels in the treatment receiving live *P. marinus*. No significant increase in PI activity was

observed in the artificial seawater treatment at any time. Non-specific PI activity in the live and killed *P. marinus* treatments also appeared to be induced at week 1 p.c., however, PI activity did not remain elevated at weeks 3 and 5 p.c. Rather, PI activity decreased to near baseline levels. Additionally, non-specific PI activity at weeks 3 and 5 p.c. for the live *P. marinus* treatment dropped below the baseline.

Introduction

Perkinsus marinus is a protozoan pathogen of the eastern oyster, *Crassostrea virginica*, and is the causative agent in the disease known as Dermo (Andrews, 1988). It has been the most important oyster pathogen in the Chesapeake Bay since the late 1980's and is responsible for massive mortalities in this species (Andrews, 1996; Burreson and Calvo, 1996). In addition to Dermo, other factors such as overharvesting and destruction of oyster reefs have resulted in a dramatic decline in Virginia oyster harvests to less than 10,000 bushels per year in the mid-1990's (Burreson and Calvo, 1996).

One approach to rejuvenating oyster populations has been the selective breeding of disease resistant oysters. For decades oysters have been selected for resistance to *Haplosporidium nelsoni*, the causative agent in MSX (Haskin and Ford, 1979; Ewart et al., 1988; Matthiessen et al., 1990; Matthiessen and Davis, 1991). Despite the success in producing generations of MSX-resistant oysters, co-resistance to Dermo has been not been observed with any consistency (Chintala and Fisher, 1989; Chintala and Fisher, 1991; Burreson, 1991). Recently, however, an F4 generation of MSX-resistant oysters produced at the Virginia Institute of Marine Science has exhibited high growth and survival and low Dermo intensity (Calvo, Ragone-Calvo, and Burreson, pers. comm.). Although there are currently no genetic markers for disease resistance, there is evidence to support heritability of resistance (Ford and Tripp, 1996; Gaffney and Bushek, 1996).

The biochemical and physiological factors contributing to MSX and Dermo resistance are not known. Valiulis (1973) suggested there to be a common mechanism of resistance to both diseases, however, there has been no evidence thus far to support this (Ford and Tripp, 1996). One approach to understanding the mechanism(s) of resistance to MSX and Dermo has been to examine cellular and humoral defenses of resistant oysters. The cellular and humoral defenses of the eastern oyster are well-documented (Anderson, 1996; Chu, 1988; Feng, 1988; Ford and Tripp, 1996). For example, Ford (1986) observed a dramatic decline in hemolymph protein concentration in MSX-susceptible oysters with a concurrent increase in MSX intensity when compared to MSX-resistant oysters. Ford suggested that such a depression in hemolymph proteins was due to long-term systemic parasitism and that their loss during *H. nelsoni* infections reflected their overall importance to the health of the oyster. Chintala and Fisher (1989) noted an association of lower susceptibility with higher serum lectin concentrations in MSX-resistant oysters compared to native oysters, although no precise causative relationship was posed. Ling (1990) observed an increase in hemocyte numbers (hemocytosis) in MSX-resistant oysters compared to MSX-susceptible oysters when challenged with *H. nelsoni*. With respect to Dermo, Chu and La Peyre (1993) compared three separate stocks of oysters to determine differences in specific humoral and cellular responses to *P. marinus* challenge. They observed the lowest cumulative mortality and the highest serum protein and lysozyme concentration in the Deep Water Shoals (DWS) stock (James River, VA). Also, the percentage of granulocytes in the DWS stock was greater than that in the other groups. Chu and La Peyre speculated that this difference might have

provided the DWS stock with a physiological advantage over the other groups and thus were less susceptible to infection.

Another approach in determining the factors associated with disease resistance is to examine virulence factors of the pathogen and look for host defense factors which would counteract them. The culturing of *P. marinus* (La Peyre et al., 1993) led to the subsequent detection of extracellular serine proteases in the culture medium (La Peyre and Faisal, 1995; La Peyre et al., 1995; La Peyre et al., 1996) and in infected oysters (Ottinger et al., pers. comm.). These proteases have been shown to have detrimental effects on oyster defense mechanisms (Garreis, 1996; Anderson, 1999). In this vein, Faisal et al. (1998) and Oliver et al. (1999) postulated that protease inhibitors might be important factors involved in host humoral defense and Dermo-resistance. Protease inhibitors have been detected in a number of marine molluscs (Armstrong and Quigley, 1992) and have been implicated in disease resistance in the snail, *Biomphalaria glabrata*, (Fryer et al., 1991). They have also been suggested as important factors in disease resistance in teleosts (Ellis, 1991; Freedman, 1991).

Work by Oliver et al. (M.S. thesis, chapter 2) has implicated low molecular weight (8-10 kDa) protease inhibitors as important factors in Dermo-resistance in selectively bred MSX- and Dermo-resistant oysters. During field challenges of three stocks of oysters, one Dermo-resistant and two Dermo-susceptible, Oliver and Kaattari (M.S. thesis, chapter 3) observed lower protease inhibitory (PI) activity in more heavily *P. marinus*-infected oysters compared to the less infected oysters. Furthermore, as *P. marinus* infections intensified, PI activity was observed to decrease significantly in the susceptible stocks but not in the resistant stock. The reason for the decreased PI activity

in more heavily infected oysters was unclear. However, Oliver and Kaattari postulated that PI activity might be directly compromised by *P. marinus* proteases through proteolytic digestion or indirectly through suppression of protease inhibitor expression. Furthermore, Oliver et al. noted the possibility of outside pathogens having adverse effects on PI activity. Due to these complications, there is a need to describe the dynamics of PI activity over time, specifically with respect to *P. marinus* infections alone.

The purpose of this study was to infect susceptible oysters with *P. marinus* and monitor PI activity over the course of five weeks to describe PI activity dynamics.

Materials and Methods

Oysters

Two-hundred market sized oysters (76-100 mm in shell height) were acquired from the Pemaquid Oyster Company, Inc. (Pemaquid, ME) in January 1999. Oysters were acclimated to 25 ppt salinity over the course of three weeks in using Forty Fathoms Artificial Sea Water in ambient filtered York River water. Oysters were held in two, sixty gallon tanks and water was changed every other day. Water was filtered using two, one micron pore size polypropylene filters (Filter Equipment Co., Inc., Wall, NJ). Oysters were fed a diet of 0.3 g algae paste/oyster per day (*Thalassosira weissflogii*) and *Isochrysis galbana* and *Chaetoceros calcitrans* at a density of 60,000 – 100,000 cells/ml per day before and during the experiment. Oysters were maintained in this manner until the initiation of the experiment. Upon arrival, a subsample of 30 oysters was taken for

determination of *P. marinus* infection by RFTM (Ray, 1952, 1966). All oysters were diagnosed negative for *P. marinus*.

P. marinus Culture and Propagation for Challenge

A culture of *P. marinus*, isolate P-1, was obtained through the generous donation by Dr. Jerome La Peyre (Louisiana State University). *P. marinus* was cultured in JLORDP-1 chemically defined medium according to the methods described by La Peyre et al. (1993). Cultures were grown to a density of 1×10^6 viable cells/ml and cells were washed, stained, and counted according to the methods described by La Peyre et al. (1993). A concentration of 1×10^6 viable cells/ml was prepared in sterile-filtered (0.2 μ m Costar bottle top filter) artificial seawater (Forty Fathoms Marine Mix Artificial Sea Water, 22 g/L) and kept at 17°C.

Formalin-treated and heat-inactivated *P. marinus* cells were generated using the following protocol. Cultures were grown to a density of 1×10^6 viable cells/ml and harvested. Cells were centrifuged at 400 x g at 25°C for 20 minutes. Supernatant was decanted and cells were resuspended in 10 ml of filter-sterilized phosphate buffered saline (PBS) (pH 7.4). Cells were passed through a 10 ml syringe with an 18 gauge needle to disaggregate cells. Viable cells were stained with neutral red and counted using a hemocytometer. Cells were centrifuged again at 400 x g at 25°C for 20 minutes. Supernatant was decanted and cells were resuspended in 10 ml of 10% buffered formalin phosphate. Cells were incubated overnight at 4 °C on an end-over-end rotator. Following the incubation, cells were centrifuged at 400 x g at 25°C for 20 minutes and resuspended in 10 ml of PBS. This washing procedure was performed two additional times.

Following the last wash, viable cells were counted again as previously described. The cell suspension volume was adjusted with PBS to attain a density of 1×10^6 viable cells/ml. Cells were then heat inactivated in a water bath for 30 minutes at 56 °C. Cells were washed three times via centrifugation and resuspension as previously described. Following the final centrifugation cells were resuspended in sterile artificial seawater so as to attain a density of 1×10^6 viable cells/ml.

Protease Inhibitory Activity

Protease inhibitory activity within oysters was detected and quantified using the Hide Powder Azure as a substrate as detailed by Bender et al. (1992). The protocol was modified for microtiter plate assays. First, *P. marinus* extracellular proteins (ECP) containing serine proteases were derived from culture supernatants as described by La Peyre et al. (1995). A standard curve of *P. marinus* ECP was generated by assessing undiluted *P. marinus* protease and two-fold dilutions (i.e., 1:4, 1:8, 1:16, 1:32, 1:64, and 1:128) in a 96 well microtiter plate (Costar). All dilutions employed in the standard curve were made using cold (4°C) artificial seawater (Forty Fathoms Marine Mix Artificial Sea Water, 22 g/L) and were kept on ice. Four microliters of each dilution from the standard curve was added to wells in triplicate followed by 50 µl of room temperature (RT) artificial sea water. Fifty-four microliters of artificial seawater at RT was added as a blank. Four microliters of undiluted protease was added to separate wells in triplicate followed by 50 µl of low molecular weight (LMW) oyster plasma containing PI activity. Negative controls consisted of 54 µl of plasma only. A protease control consisted of 4 µl undiluted protease plus 50 µl RT artificial seawater. The blank for the protease control

consisted of 54 μ l of RT artificial seawater only. Mixtures of protease and plasma were incubated at RT for 15 minutes on a shaker. Following incubation, 100 μ l of substrate suspension (Hide Powder Azure, 0.02 g/ml, 150 mM Tris, 30 mM CaCl_2 , 0.05% Brij, 20% sucrose, pH 7.5) was added to each well. Plates were incubated at 37°C on a shaker for 3 hours. After incubation, 100 μ l of cold artificial seawater was added to each well to stop the reaction. Plates were centrifuged at 2700 x g for 15 minutes at 4°C. One hundred microliters of supernatant were removed from each well and transferred to another 96 well microtiter plate. Plates were read at 540 nm on a Titertek® Multiskan® MCC/340 plate reader (Labsystems, Finland) using DeltaSoft 3 software (BioMetallics, Inc., Princeton, NJ). Based on the protease standard curve, reduction in A_{540} due to the inhibition by LMW *C. virginica* plasma was calculated and expressed as ng *P. marinus* protease inhibited/50 μ l LMW plasma.

Challenge Procedure

The experiment was initiated by notching 60 oysters using a radial saw. Notches were made near the adductor muscle so as to make a small exposure to the mantle cavity. Individual oysters were placed in 800 ml plastic beakers and acclimated for one week. Temperature and salinity of the water was maintained at 23°C +/- 2°C and 25 ppt, respectively, throughout the experiment.

The oysters were separated into three groups of twenty and acclimated. After one week of acclimation, prebleeds of oysters were procured in order to establish baseline of PI values. Oysters were bled via the adductor muscle using a 1 cc syringe and a 1 inch, 25 gauge needle. Oyster hemolymph was immediately transferred to a 1.5 ml centrifuge

tube on ice. Care was taken in rinsing the needle in deionized water between bleeds. Hemolymph was centrifuged at 400 x g for 10 minutes at 4°C. The cell-free portion, or plasma, was then centrifuged in a Microcon-10 apparatus (Amicon Inc., Beverly, MA) to fractionate low molecular weight plasma (<10 kDa). The filtrate containing less than 10 kDa molecules was stored at -20°C until further use.

Three weeks later each group of oysters was challenged with either 1×10^6 live *P. marinus* cells, 1×10^6 formalin-treated/heat-inactivated *P. marinus* cells, or 1 ml of sterile artificial seawater via mantle cavity injections following the procedure described by Bushek et al. (1997). Oysters were challenged every other day for a total of 4 days.

Oyster hemolymph was sampled at 1, 3, and 5 weeks post-challenge (p.c.) using the methods previously described. Following the last bleed at week 5 p.c. all oysters were sacrificed for diagnosis of *P. marinus* infections.

Protein Determination

Protein determinations were performed for all oysters throughout the experiment. Protein concentration (mg/ml) was determined using the BCA Protein Assay Kit (Pierce, Rockford, IL).

Disease Diagnosis

Diagnosis of *P. marinus* infections was performed using Ray's Fluid Thioglycollate Method (RFTM) (Ray, 1952, 1966) and infections were scored according to the method described by Mackin (1962).

Results

Disease Diagnosis

All oysters were diagnosed for *P. marinus* infections after the final bleed (data not shown). The P-1 treatment showed 63% prevalence of *P. marinus* with an average intensity of 0.63 on the Mackin scale.

Protease Inhibitory Activity

The analysis of PI activity for each treatment is presented in Figures 1b and 1c. Specific PI activity increased significantly ($p < 0.001$) for both live and killed *P. marinus* treatments at week 1 (Figure 1b). Specific PI activity decreased at week 3 and stabilized at week 5 in the live *P. marinus* treatment to just above the baseline. In contrast to the live *P. marinus* treatment, specific PI activity in the killed *P. marinus* treatment remained significantly higher than the baseline at weeks 3 and 5 (Figure 1b). No significant change in specific PI activity relative to the baseline was observed in the treatment receiving artificial seawater.

There was considerable variation in baseline levels of non-specific PI activity as the P-1 treatment had the highest baseline level PI followed by ASW and P-1C treatments (Figure 1c). This variability in non-specific PI activity has been consistently observed in a variety of studies (Oliver et al., M.S. thesis, chapter 2; Oliver and Kaattari, M.S. thesis, chapter 3). In the P-1 treatment, PI increased at week 1 p.c. and then steadily decreased

at weeks 3 and 5. The PI activity at weeks 3 and 5 was lower than the baseline with week 5 PI activity being significantly lower ($p < 0.001$). The P-1 control treatment (P-1C) also exhibited a similar trend as the P-1 treatment. Non-specific PI activity at week 1 was significantly higher ($p < 0.001$) than the baseline with a marked decrease to near baseline levels at week 3. In contrast to the P-1 treatment, P-1C PI activity did not continue to decrease at week 5. Rather PI activity remained close to the level of week 3. Furthermore, the PI activity at weeks 3 and 5 were slightly higher than the baseline, but they were not significant. Non-specific PI activity in the artificial seawater control (ASW) exhibited a similar trend as the first two treatments. There was an increase in PI activity at week 1 with PI activity decreasing at weeks 3 and 5. Similar to the P-1C treatment, week 3 PI activity dropped to near the baseline level, however, it continued to decrease at week 5. Interestingly, the PI activity at week 5 was lower than the baseline that was also seen in the P-1 treatment.

Changes in PI activity relative to the baseline of each treatment were plotted in Figures 2a and 2b. In this way the treatment-specific changes in PI could be more easily seen.

Plasma Protein Concentration

All treatments exhibited decreases in plasma protein concentration during the experiment (Figure 1a). Decreases in plasma protein concentration might be explained by the change in food quality between the time oysters were received and the final bleed. Oysters were only fed three algal types during the experiment and may have suffered nutritional deprivation as reflected in the plasma protein levels.

Discussion

Eastern oysters are known to have numerous humoral defense factors (Chu, 1988; Anderson, 1996; Ford and Tripp, 1996). Some of the best studied are the lectins (McDade and Tripp, 1967a; Olafsen, 1988; Vasta, 1991) and lysosomal enzymes (McDade and Tripp, 1967b; Cheng and Roderick, 1975; Chu and La Peyre, 1989). Recently, protease inhibitors have received attention as important defense factors during *P. marinus* infections (Faisal et al. 1998; Oliver et al. 1999a) as well as a general molluscan defense mechanism (Armstrong and Quigley, 1992; Bender and Bayne, 1992, 1996; Thorgersen et al., 1992; Elsayed et al., 1999). Their role in Dermo-resistance has also been suggested (Oliver et al., 1999b). Oliver et al. (1999b) examined field oysters exhibiting a wide range of Dermo-resistance and found that low molecular weight (LMW) protease inhibitory (PI) activity was positively correlated with survival. Additionally, Dermo intensity as measured on the Mackin scale was negatively correlated with PI. The latter correlation was significant; however, it was unclear whether *P. marinus* was the lone factor in altering PI. One reason Oliver et al. offered was the possibility that other pathogens might participate in suppressing PI. Furthermore, Oliver et al. could not determine if and how PI activity varied during the course of a *P. marinus* infection. Therefore, this study was intended to elucidate the dynamics of LMW PI activity during a controlled experimental infection with *P. marinus*.

Results of the *P. marinus* challenge experiment showed a variation in the resting or baseline levels of specific and non-specific PI activity in susceptible oysters. Due to the variation in baseline PI between each treatment, comparison of absolute PI activity between treatments after challenge could not be made. Instead, the change in PI activity from the baseline was made for each treatment and compared. An increase in specific PI activity was observed after challenge at week 1 with live *P. marinus* (P-1) and formalin-treated/heat-inactivated *P. marinus* (P-1C). No significant increase in specific PI activity was observed in the artificial seawater treatment. *P. marinus* cells possess membrane-associated proteases as well as having the ability to secrete proteases into the extracellular medium (La Peyre et al., 1995; M. Faisal, pers. comm.). Therefore, it would not seem surprising that PI would be induced even in the presence of formalin fixed *P. marinus* cells. Thus it would appear that PI is inducible and the induction may be antigen-specific to protease.

Non-specific PI activity exhibited different dynamics than specific PI activity. Like specific PI activity, non-specific PI activity increased in both the live and killed *P. marinus* treatments at week 1. The increase in the P-1 challenge was not significant and the increase in the P-1C challenge was significant. However, in contrast to specific PI activity, non-specific PI activity decreased below baseline levels in the P-1 challenge and decreased to near baseline levels in P-1C challenge at weeks 3 and 5. Challenges with artificial seawater also appear to have elicited a response in non-specific PI activity. It is possible that oysters may be hypersensitive to the injection process and seawater alone can elicit a PI activity response. It is otherwise unclear why such an induction of PI activity would occur in this treatment.

It is interesting to note the decline in non-specific PI activity in all treatments observed herein. One reason for the decline in non-specific PI activity may be that plasma protein concentrations also decreased. Previous studies have noted the association between plasma protein and disease intensity (Ford, 1986; Chu and La Peyre, 1993). Specifically, inverse associations between plasma protein concentrations and susceptibility have been observed. This inverse association was not observed by Oliver and Kaattari (M.S. thesis, chapter 3) with respect to non-specific PI activity and Dermo infection. Rather, Oliver and Kaattari found no association between Dermo intensity and plasma protein concentration since there was no significant difference in plasma protein at any scoring of the RFTM scale. They suggested that PI activity was not coupled to plasma protein dynamics. This assertion is supported by the observation herein that non-specific PI activity does not appear to exhibit any predictable pattern with respect to plasma protein concentration. These findings challenge the appropriateness of standardizing PI activity to plasma protein concentration. Changes in plasma protein concentration may bias specific PI activity as lower protein may skew PI activity higher. Conversely, higher protein may skew PI activity lower.

The continual decrease in non-specific PI activity in the P-1 treatment and the stabilization of PI in the P-1C treatment at weeks 3 and 5 suggests that PI may be vitally important in controlling initial infections. Although it appears naïve oysters can respond positively to *P. marinus* challenges in the short term, it appears that oysters are unable to maintain PI beyond baseline PI activity and that eventually *P. marinus* is able to overcome host expression of inhibitor. The mechanism of overcoming host inhibitor is not known. It is possible that there may be a competitive over-expression of protease

versus inhibitor or there may be a direct suppressive effect on inhibitor expression. The continual decrease in non-specific PI activity in the ASW treatment from week 3 to 5 p.c. cannot be explained. However, non-specific PI activity in the ASW treatment initially appears to follow a similar pattern to the P-1C treatment as PI activity dropped from week 1 to 3 to near the baseline level. This is in contrast to the P-1 treatment where non-specific PI activity dropped below baseline the level at week 3 p.c. Although it is tempting to conclude that live *P. marinus* secretory products continually scavenged host inhibitor over time in the P-1 treatment, the precipitous drop in PI at week 5 p.c. in the ASW treatment precludes this conclusion.

The induction of humoral factors in molluscs exposed to non-self antigens or pathogens is not unprecedented. Anderson (1981) observed the induction of hemolytic activity in the clam, *Mercenaria mercenaria*, toward mammalian erythrocytes and posed its protective function in the host. Olafsen et al. (1992) observed the induction of agglutinin activity in Pacific oysters (*Crassostrea gigas*) after challenging with *Vibrio anguillarum* and suggested that agglutinins are involved in enhancing bacterial clearance. Montes et al. (1996) observed the induction of a 225 kDa protein believed to be important in defense against *Perkinsus* spp. Additional studies have attempted to associate humoral factors with disease resistance in oysters; however, there has yet to be conclusive evidence that certain humoral factors confer resistance. Ford (1986) compared hemolymph proteins from resistant and susceptible oysters exposed to MSX, but found that SDS-PAGE banding patterns could not be correlated with resistance. Chintala et al. (1991) observed significantly higher agglutination titers for *Vibrio cholerae* in MSX-resistant oysters, however, Chintala et al. (1994) found no correlation between parasite

densities and agglutination titers and concluded that serum agglutinins played no role in defense against *H. nelsoni* or *P. marinus*.

All oysters were diagnosed for *P. marinus* infections after the final bleed. The P-1 treatment had an average *P. marinus* infection of 0.63 on the Mackin scale. Thus, it appears that only light infections with *P. marinus* are required to reduce non-specific PI activity. It is unclear how PI activity would behave beyond light infections. Continual reduction in PI activity to undetectable levels might occur, however, extended monitoring of PI activity over time is needed to confirm this. No significant difference in non-specific PI activity was detected between uninfected and lightly infected oysters. This seems to contradict the observation that non-specific PI activity was dramatically lower in the P-1 treatment at weeks 3 and 5. The lower sample size in the lightly infected group (N = 7) versus the uninfected group (N = 31) may account for the absence of a statistical difference.

It is interesting to note that the increase in non-specific PI activity in the P-1 treatment at week 1 was approximately 25% while the increase in the P-1C treatment was over 100%. One reason that may account for the differential increase in PI activity at week 1 is that in the P-1 treatment the pathogen is continually expressing protease. This would lead to a removal of unbound or free host inhibitor resulting in less of an increase in PI compared to the P-1C treatment. The observation that non-specific PI activity was induced in oysters injected with artificial seawater (ASW) would lead to the conclusion that the PI response is not antigen-specific. To determine whether the PI induction is antigen-specific to protease, *P. marinus* membranes would need to be stripped of protease and an additional control group receiving no injection would need to be included.

The variations in PI during this experiment is reflective of only a short, albeit concentrated, dose of *in vitro* cultured *P. marinus* cells over the course of one week. It should be noted that oysters in the wild are not likely to be exposed to such a density of cells in such a short time span and thus, the PI may not vary as widely due to *P. marinus* infections as observed herein. This does not preclude the observation that PI may exhibit changes of the same magnitude in the wild. One reason for this would be the presence of other pathogens capable of altering PI. The dosage used in this experiment was determined according to previous reports detailing the numbers of cells required to establish an infection (Chu, 1996; Chu, 1997; Bushek et al., 1997). Furthermore, the establishment of only light infections after five weeks p.c. is consistent with the findings of these investigators.

This study has demonstrated the variability in PI in naïve, susceptible oysters and how PI changes after challenges with live and killed *P. marinus*. Although the data is not conclusive, it suggests that *P. marinus* secretory products can exert deleterious effects on the host's ability to maintain PI throughout an infection. The long-term role of PI in oyster humoral defense against *P. marinus* is unclear. In the short term, it appears that PI is incapable of eliminating *P. marinus* infections. Perhaps a longer infection study employing an additional group of naïve Dermo-resistant oysters might further elucidate the role of PI in controlling *P. marinus* infections.

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References

- Anderson, R. S. 1981. Inducible hemolytic activity in *Mercenaria mercenaria* hemolymph. *Dev. Comp. Immunol.* **5**:575-585.
- Anderson, R. S. 1996. Interactions of *Perkinsus marinus* with humoral factors and hemocytes of *Crassostrea virginica*. *J. Shellfish Res.* **15**:127-134.
- Anderson, R. S. 1999. *Perkinsus marinus* secretory products modulate superoxide anion production by oyster (*Crassostrea virginica*) haemocytes. *Fish Shellfish Immunol.* **9**:51-60.
- Andrews, J. D. 1988. Epizootiology of the disease caused by the oyster pathogen *Perkinsus marinus* and its effect on the oyster industry. *Am. Fish. Soc. Spec. Publ.* **18**:47-63.
- Andrews, J. D. 1996. History of *Perkinsus marinus*, a pathogen of oysters in Chesapeake Bay 1950 – 1984. *J. Shellfish Res.* **15**:13-16.
- Armstrong, P. B. and Quigley, J. P. 1992. Humoral immunity: α_2 -macroglobulin activity in the plasma of mollusks. *Veliger.* **35**:161-164.
- Bender, R. C., Fryer, S. E., and Bayne, C. J. 1992. Proteinase inhibitory activity in the plasma of a mollusc: Evidence for the presence of α -macroglobulin in *Biomphalaria glabrata*. *Comp. Biochem. Physiol.* **102B**(4):821-824.
- Bender, R. C., and Bayne C. J. 1996. Purification and characterization of a tetrameric α_2 -macroglobulin proteinase inhibitor from the gastropod mollusc *Biomphalaria glabrata*. *Biochem. J.* **315**:893-900.

- Burreson, E. 1991. Effects of *Perkinsus marinus* infection in the eastern oysters, *Crassostrea virginica* (Gmelin, 1791): I. Susceptibility of native and MSX-resistant stocks. *J. Shellfish Res.* 10:417-423.
- Burreson, E. M. and Ragone Calvo, L. M. 1996. Epizootiology of *Perkinsus marinus* disease of oysters in Chesapeake Bay, with emphasis on data since 1985. *J. Shellfish Res.* 15:17-34.
- Bushek, D., Allen, S. K., Jr., Alcox, K. A., Gustafson, R. G., and Ford, S. E. 1997. Response of *Crassostrea virginica* to *in vitro* cultured *Perkinsus marinus*: Preliminary comparisons of three inoculation methods. *J. Shellfish Res.* 16(2):479-485.
- Cheng, T. C. and Rodrick, G. E. 1975. Lysosomal and other enzymes in the hemolymph of *Crassostrea virginica* and *Mercenaria mercenaria*. *Comp. Biochem. Physio.* 52B:443-447.
- Chintala, M. M. and Fisher, W. S. 1989. Comparison of oyster defense mechanisms for MSX-resistant and –susceptible stocks held in Chesapeake Bay. *J. Shellfish Res.* 8:846-847.
- Chinatala, M. M. and Fisher, W. S. 1991. Disease incidence and potential mechanisms of defense for MSX-resistant and –susceptible eastern oysters held in Chesapeake Bay. *J. Shellfish Res.* 10:439-443.
- Chinatala, M. M., Ford, S. E., Fisher, W. S., and Ashton-Alscox, K. A. 1994. Oyster serum agglutinins and resistance to protozoan parasites. *J. Shellfish Res.* 13:115-121.
- Chu, F.-L. 1988. Humoral defense factors in marine bivalves. *Am. Fish. Soc. Spec.*

- Publ.* 18:178-188.
- Chu, F. L. and La Peyre, J. F. 1989. Effect of environmental factors and parasitism on hemolymph lysozyme and protein of American oysters (*Crassostrea virginica*). *J. Invertebr. Pathol.* 54:224-232.
- Chu, F.-L. and La Peyre, J. F. 1993. Development of disease caused by the parasite, *Perkinsus marinus*, and defense-related hemolymph factors in three populations of oysters from the Chesapeake Bay, USA. *J. Shellfish Res.* 12:21-27.
- Ellis, A. E. 1991. Inhibition of the *Aeromonas salmonicida* extracellular protease by α_2 -macroglobulin in the serum of rainbow trout. *J. Fish Dis.* 14:265-277.
- Elsayed, E. E., McLaughlin, S. M. and Faisal, M. 1999. Effects of neoplasia on plasma protease inhibitory activities in the softshell clam, *Mya arenaria*. *Comp. Biochem. Physiol.* In press.
- Ewart, J. W., Cole, R., and Tinsman, J. 1988. Growth and survival of hatchery produced MSX resistant oyster stocks in Delaware Bay. *J. Shellfish Res.* 7:543.
- Faisal, M., MacIntyre, E. A., Adham, K. G., Tall, B. D., Kothary, M. H., and La Peyre, J. F. 1998. Evidence for the presence of protease inhibitors in eastern (*Crassostrea virginica*) and Pacific (*Crassostrea gigas*) oysters. *Comp. Biochem. Physiol.* 121B:161-168.
- Feng, S. Y. 1988. Cellular defense mechanisms of oysters and mussels. *Am. Fish. Soc. Spec. Publ.* 18:153-168.
- Ford, S. E. 1986. Comparison of hemolymph proteins between resistant and susceptible oysters, *Crassostrea virginica*, exposed to the parasite, *Haplosporidium nelsoni* (MSX). *J. Invertebr. Pathol.* 47:283-294.

- Ford, S. E. and Tripp, M. R. 1996. Chapter 17: Diseases and Defense Mechanisms. *In* "The Eastern Oyster, *Crassostrea virginica*" (V. S. Kennedy, R. I. E. Newell, and A. F. Eble, Eds.), pp.581-660. Maryland Sea Grant College, College Park, Maryland.
- Freedman, S. J. 1991. The role of α_2 -macroglobulin in furunculosis: a comparison of rainbow trout and brook trout. *Comp. Biochem. Physiol.* **98B**:549-553.
- Fryer, S. E., Bender, R. C. and Bayne, C. J. 1991. Proteinase inhibitory activity in the plasma of the gastropod mollusc, *Biomphalaria glabrata*. *Dev. Comp. Immunol.* **15**:S45.
- Gaffney, P. M. and Bushek, D. 1996. Genetic aspects of disease resistance in oysters. *J. Shellfish Res.* **15**(1):135-140.
- Garreis, K. A, La Peyre, J. F, and Faisal, M. 1996. The effects of *Perkinsus marinus* extracellular products and purified proteases on oyster defence parameters *in vitro*. *Fish Shellfish Immunol.* **6**:581-597.
- Haskin, H. H. and Ford, S. E. 1979. Development of resistance to *Minchinia nelsoni* (MSX) mortality in laboratory-reared and native oyster stocks in Delaware Bay. *Mar. Fish. Rev.* **41**:54-63.
- Haskin, H. H. and Ford, S. E. 1987. Breeding for resistance in molluscs. *In*: Proceedings of World Symposium on Selection, Hybridization, and Genetic Engineering in Aquaculture, Bordeaux, 27-30 May 1986, Vol. II. Heenemann, Berlin. pp. 432-441.
- La Peyre, J. F., Faisal, M., and Burreson, E. M. 1993. *In vitro* propagation of the protozoan *Perkinsus marinus*, a pathogen of the eastern oyster, *Crassostrea virginica*. *J. Euk. Microbiol.* **40**:305-310.

- La Peyre, J. F. and Faisal, M. 1995. *Perkinsus marinus* produces extracellular Proteolytic factor(s). *Bull. Eur. Assoc. Fish Pathol.* 15:1-4.
- La Peyre, J. F., Schafhauser, D. Y., Rizkalla, E. H., and Faisal, M. 1995. Production of serine proteases by the oyster pathogen *Perkinsus marinus* (Apicomplexa) *in vitro*. *J. Euk. Microbiol.* 42:544-551.
- La Peyre, J. F., Yarnall, H. A., and Faisal, M. 1996. Contribution of *Perkinsus marinus* Extracellular products in the infection of Eastern oysters (*Crassostrea virginica*). *J. Invertebr. Path.* 68:312-313.
- Levine, N. D. 1978. *Perkinsus* gen. N. and other new taxa in the protozoan phylum Apicomplexa. *J. Parasitol.* 64:549.
- Ling, Y.-J. 1990. Cellular and humoral responses of resistant and susceptible oysters, *Crassostrea virginica*, to the infection of *Haplosporidium nelsoni* (MSX). M.S. Thesis. University of Connecticut, Avery Point. 102 pp.
- Mackin, J. G. 1962. Oyster disease caused by *Dermocystidium marinum* and other microorganisms in Louisiana. *Publ. Inst. Marine Sci. Univ. Texas.* 7:132-229.
- Matthiessen, G. C., Feng, S. Y., and Leibovitz, L. 1990. Patterns of MSX (*Haplosporidium nelsoni*) infection and subsequent mortality in resistant and susceptible strain of the eastern oyster, *Crassostrea virginica* (Gmelin, 1791), in New England. *J. Shellfish Res.* 9:359-365.
- Matthiessen, G. C. and Davis, J. P. 1991. Growth, disease resistance and survivorship of diploid and triploid eastern oysters in an MSX environment. *J. Shellfish Res.* 10:298-299.

- McDade, J. E. and Tripp, M. R. 1967a. Lysozyme in the hemolymph of the oyster, *Crassostrea virginica*. *J. Invertebr. Pathol.* 9:51-535.
- McDade, J. E. and Tripp, M. R. 1967b. Mechanism of agglutination of red blood cells by oyster hemolymph. *J. Invertebr. Pathol.* 9:523-530.
- Montes, J. F., Durfort, M., and Garcia-Valero, J. 1996. Cellular defence mechanism of clam *Tapes semidecussatus* against infection by the protozoan *Perkinsus* sp. *Cell Tissue Res.* 279: 529-538.
- Olafsen, J. A. 1988. Role of lectins in invertebrate humoral defense. *In: Disease Processes in Marine Bivalve Molluscs*. W. S. Fisher (ed.). *Amer. Fish. Soc. Spec. Publ.* 18. Bethesda, Maryland, pp. 189-205.
- Olafsen, J. A., Fletcher, T. C., and Grant, P. T. 1992. Agglutinin activity in Pacific oyster (*Crassostrea gigas*) hemolymph following in vivo *Vibrio anguillarum* challenge. *Dev. Comp. Immunol.* 16:123-138.
- Oliver, J. L., Lewis, T. D., Faisal, M., and Kaattari, S. L. 1999. Analysis of the effects of *Perkinsus marinus* proteases on plasma proteins of the eastern (*Crassostrea virginica*) and Pacific oyster (*Crassostrea gigas*). *J. Invertebr. Path.* 74:173-183.
- Oliver, J. L., Gaffney, P. M., Allen, S. K., Faisal, M., and Kaattari, S. L. 1999b. Plasma of *Crassostrea* spp. possess a low molecular weight inhibitor of *Perkinsus marinus*. *J. Shellfish Res.* 18(1):319.
- Ray, S. M. 1952. A culture technique for the diagnosis of infection with *Dermocystidium marinum* (Mackin, Owen, and Collier) in oysters. *Science.* 116:360-361.

- Ray, S.M. 1966. A review of the culture method for detecting *Dermocystidium marinum*, with suggested modifications and precautions. *Proc. Natl. Shellfish Assoc.* **54**:55-69.
- Thogersen, I. B., Salvesen, G., Brucato, F. H., Pizzo, S. V. and Enghild J. J. 1992. Purification and characterization of an α_2 -macroglobulin from the mollusc *Octopus vulgaris*. *Biochem. J.* **285**:521-527.
- Valiulis, G. A. 1973. Comparison of the resistance to *Labyrinthomyxa marina* with resistance to *Minchinia nelsoni* in *Crassostrea virginica*. Ph.D. Dissertation. Rutgers University, New Brunswick, NJ. 180 pp.
- Vasta, G. R. 1991. The multiple biological roles of invertebrate lectins: their participation in nonself recognition mechanisms. *In: Phylogenesis of Immune Functions*. G. W. Warr and N. Cohen (eds.). CRC Press, Boca Raton, Florida, pp. 73-101.

Figure Legends

Figure 1a. Mean plasma protein concentration (mg/ml) during challenge experiment.

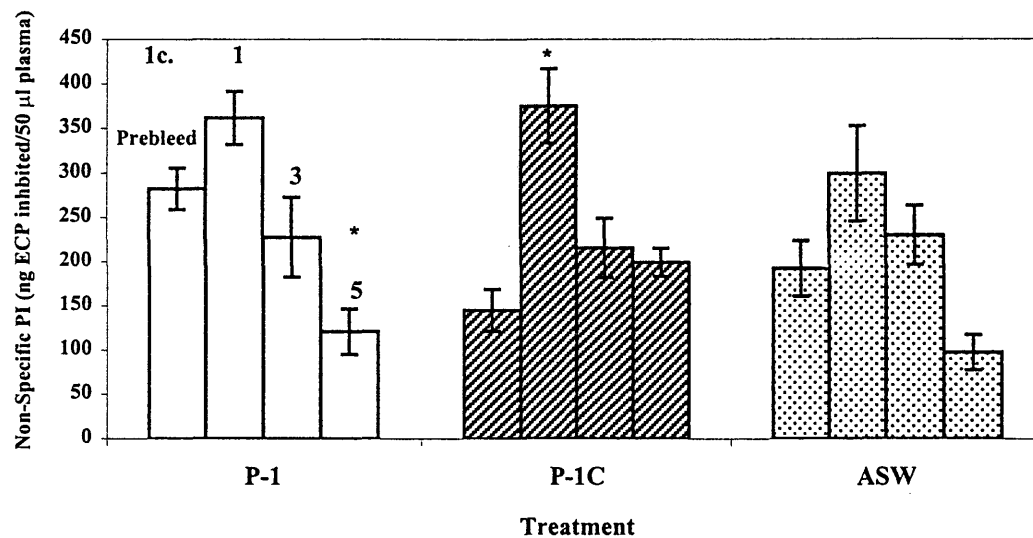
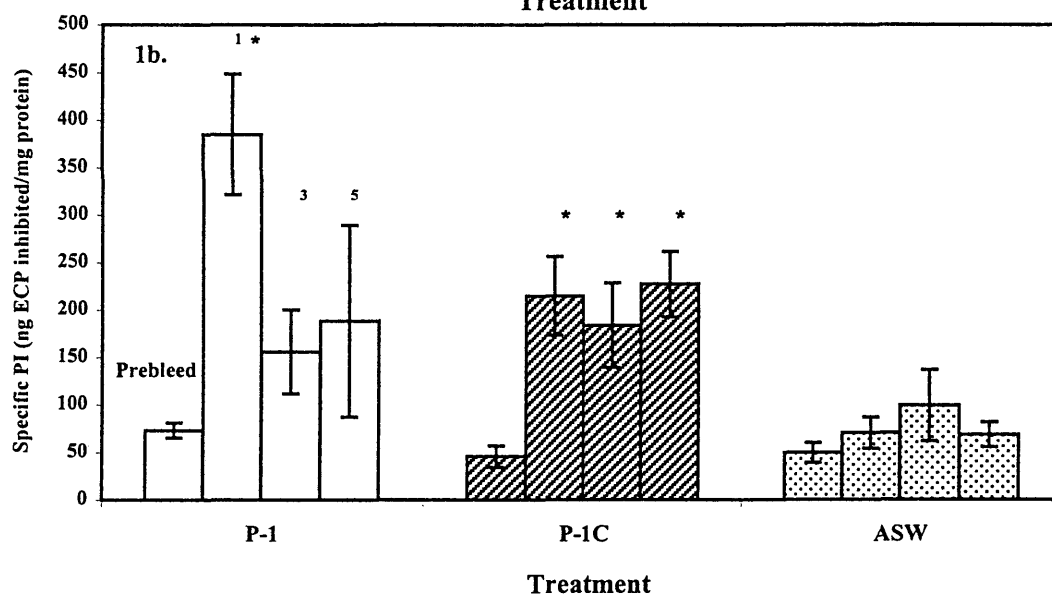
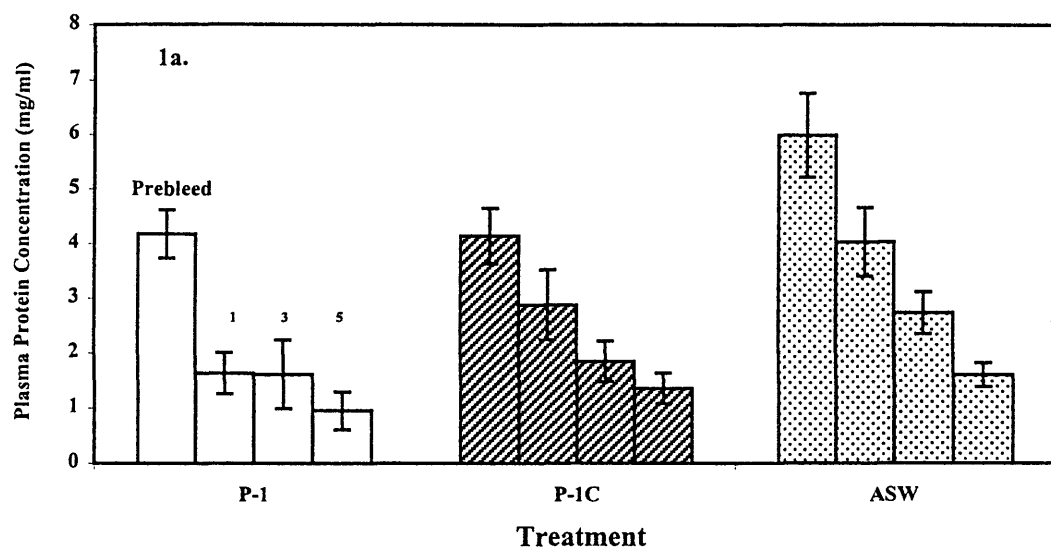
Error bars represent the standard error. Time (in weeks) post-challenge is denoted by the numbers above the bars and is the same for each treatment. Treatments defined as follows: P-1, live *P. marinus*-1 challenge; P-1C, formalin treated/heat killed *P. marinus*-1 challenge; ASW, artificial seawater challenge (22 mg/l).

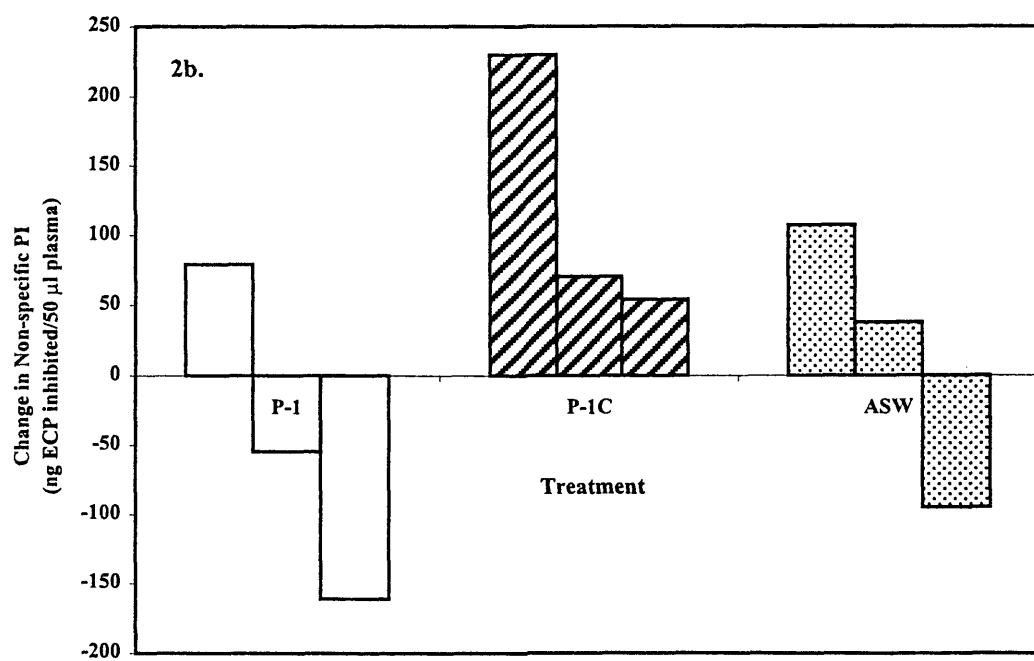
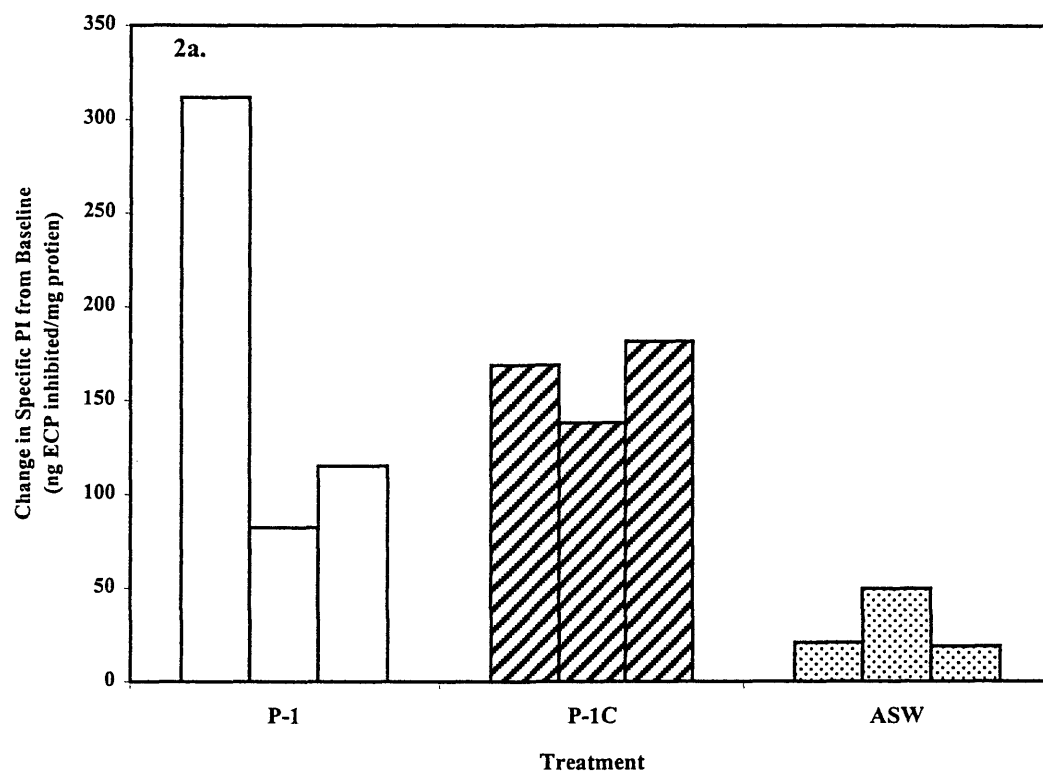
Figure 1b. Mean specific protease inhibitory (PI) activity. Asterisks denote significant differences from the prebleed ($p < 0.001$). Error bars represent the standard error. Time (in weeks) post-challenge is denoted by the numbers above the bars and is the same for each treatment.

Figure 1c. Mean non-specific protease inhibitory (PI). Asterisks denote significant differences from the prebleed ($p < 0.001$). Error bars represent the standard error. Time (in weeks) post-challenge is denoted by the numbers above the bars and is the same for each treatment.

Figure 2a. Mean change in specific PI activity from baseline. Baseline PI activity was measured 3 weeks prior to challenge. Each bar represents the mean change in PI activity at weeks 1, 3, and 5 post-challenge.

Figure 2b. Mean change in non-specific PI activity from baseline. Baseline PI activity was measured 3 weeks prior to challenge. Each bar represents the mean change in PI activity at weeks 1, 3, and 5 post-challenge.





General Discussion

This thesis explored the associations of protease inhibitors of the eastern oyster, *Crassostrea virginica*, with infections by the oyster pathogen, *Perkinsus marinus*. Specifically, protease inhibitory (PI) activity was detected in the plasma of oysters. This PI activity was determined to be of low molecular weight (< 10 kDa) and demonstrated specificity for *P. marinus* extracellular proteases. Additionally, PI activity was capable of preventing the digestion of a sensitive plasma protein target (35 kDa) in the oyster by *P. marinus* extracellular proteases. From these findings, a protective function of PI activity was postulated with respect to Dermo disease resistance.

Selectively bred families of oysters with high genetic variability and exhibiting wide ranges of resistance to Dermo were analyzed for PI activity in an attempt to discern a relationship between PI activity and susceptibility. A positive correlation was found between family survival and mean family PI activity. A significant negative correlation between Dermo intensity (Mackin scale) and PI activity was also observed. Three selected stocks of oysters, one MSX/Dermo-resistant and two Dermo-susceptible stocks, were also analyzed for PI activity and susceptibility to Dermo. No significant relationship between stock survival and PI activity was discerned. However, there was a significant negative correlation between Dermo intensity and PI activity as seen in the selectively bred families. PI activity dynamics was explored in a *P. marinus* challenge experiment. Results indicate that PI activity is inducible in oysters challenged with live

and dead *P. marinus*. It is unclear whether this induction is antigen-specific or a non-specific response to challenge.

The positive correlation between PI activity and survival in the selectively bred families of oysters lends support to the hypothesis that PI activity plays a protective role in resisting *P. marinus* infections. An additional piece of evidence that supports PI activity as important in humoral defense against *P. marinus* is the finding that PI activity negatively correlated with Dermo intensity. The results of the *in vivo P. marinus* challenge, however, were inconclusive with respect to supporting these earlier findings. It is likely that a similar challenge experiment performed over a longer time scale might lend support to the protective role of PI activity. The variability in PI activity within selectively bred families and groups of oysters make it difficult to discern to what degree PI activity alone is capable of protecting oysters from *P. marinus* infections. Some families exhibited high PI activity and low mortality, but also there were families/groups that exhibited low PI activity and low mortality. Only when all oysters are considered, irrespective of family or group, are differences in PI activity with respect to disease intensity revealed. These results, albeit encouraging, are by no means comprehensive in elucidating the mechanism(s) of disease resistance to *P. marinus*. The findings herein do not support PI activity as being a substantial mechanism in resistance to *P. marinus*. This is clear when one considers that all putatively resistant oysters challenged in the field with *P. marinus* still contracted the disease, albeit in low intensities at times. Thus, if PI activity does have a role in humoral defense, it may be through conferring tolerance rather than complete resistance to *P. marinus*. It is possible that PI activity plays only a minor role in humoral defense against *P. marinus* and the temporal nature of such a

defense factor during the disease process is unfortunately subject to speculation at this time. It is clear that further studies are warranted in order to corroborate the findings herein.

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